An examination of VEGF, its receptors and the adherens junction component, β-catenin in the development of primary varicose veins.

Submitted in partial fulfilment for a Masters in Philosophy

Royal Free and University College Medical School

University College London

September 2009

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STATEMENT OF ORIGINALITY

Statement of Originality

I, Garth Powell confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Garth Powell

ABSTRACT

Abstract

Vascular endothelial growth factor (VEGF-A) plays a central role in the maintenance of vascular reactivity and its aberrant control/function is implicated in the development of varicose veins (VVs). However, it is unclear where the problem is; with the vessels ability to produce VEGF-A or indeed an inability to mediate an appropriate response/control to VEGF-A when produced. Here, the patterns of activation of *VEGF*, its receptors and the adherens junction component**,** β-catenin were investigated in primary VV.

Samples of varicose or control greater saphenous vein (GSV) were divided into segments by anatomical position, descending from the sapheno-femoral junction (SFJ). SFJ and segmental competence were determined from duplex scan. For each segment, patterns of gene transcription (*VEGF*, *VEGF* receptors, *β-catenin*, *c-myc* and *cyclin D1*) and β-catenin protein were examined in relation to the underlying venous incompetence. Separately, release of soluble VEGFR1 (s.flt-1, *fms* like tyrosine kinase receptor 1) was investigated following an induced venous hypertension/stasis.

In VVs overall, transcription of all genes were elevated ($p<0.001$) and in VVs with SFJ incompetence ($p<0.001$). In competent VV segments (no reflux), transcription of *VEGF-A*$_{121}$ ($p<0.02$), *VEGF-A*$_{165}$ ($p<0.006$) and *VEGFR2* ($p<0.007$) were elevated. Gene transcription was unaffected by segmental position. When the SFJ was functional β-catenin protein was elevated in VVs overall ($p<0.06$), and increased with descending position from the SFJ ($p<0.01$); transcription was unaltered. Plasma s.flt-1 was markedly elevated in VVs ($p<0.001$), while cuff application induced a rapid (10 minutes, $p<0.015$) elevation in s.flt-1 in control (54.2%) which was not observed in VVs (5.5%, $p>0.1$).

SFJ incompetence was associated with an elevated in transcription of all genes examined reflecting perhaps, a later stage of disease development. In contrast, elevated transcription of VEGF receptors in segments of competent VV may be an earlier event. Disturbed β-catenin activity (possible due to VEGF) precedes vessel wall compromise in VVs, with elevated β-catenin protein potentially providing a mechanism for vessel wall remodelling. Loss of release of s.flt-1 may be important in the pathogenesis of primary VVs, by potentially mediating the action(s) of VEGF.

ACKNOWLEDGMENTS

Acknowledgments

To my wife Helena, thank you for your understanding, patience and unfaltering support through out my period of study. To my colleagues, thank you for tolerating my queries and questions and making my period of study an enjoyable one. To my supervisors, Mr Barker and Dr Hollingsworth, thank you for your support, encouragement and guidance without which this thesis would not have been possible. To Dr Marilena Loizidou, thank you for supporting the submission of my thesis and helping me to finally realise this achievement.

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ABBREVIATIONS

Abbreviations

AJ	adherens junction
Akt	protein kinase B
AOI	area of interest
AP-1	activator protein1
AP-2	activator protein 2
arg	arginine
ARNT	aryl hydrocarbon receptor nuclear translocator
asp	aspartic acid
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
Ca^{2+}	calcium
CamKII	calmodium-dependant protein kinase II
CCND1	cyclin D1
CEAP	clinical etiology anatomy pathology
COOH	carboxyl group
CREB	cAMP response element binding protein
CTNNB1	β-catenin
CVD	chronic venous disease
CVI	chronic venous insufficiency
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
Dsh	Dishevelled
EC	endothelial cell
ECM	extra-cellular matrix
ELISA	enzyme linked immunoassay
eNOS	endothelial nitric oxide synthase
ERE	oestrogen response element
flt-1	*fms* like tyrosine kinase receptor (VEGFR1)
Fz	Frizzled receptor
GAP-3	glyceraldehyde - 3 phosphate dehydrogenase (GAPDH)
glu	glutamic acid
GSK-3β	glycogen synthase 3β
GSV	greater saphenous vein
HIF-1	hypoxia inducible factor 1
his	histidine
HRE	hypoxia response element
HRP	horse radish peroxidase
HSPG	heparin sulphate proteoglycans
HuR	hypoxia induced stability factor
ICAM-1	intracellular adhesion molecule
IL-8	interleukin 8
IOD	integrated optical density

IRES	internal ribosome entry site
KDR	kinase domain receptor (VEGFR2)
LXR	liver X receptor
lys	Lysine
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemotactic protein
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
MMP	matrix metalloproteases
mRNA	message RNA
MYC	c-myc
NRP1	Neuropilin receptor 1
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGH_2	prostaglandin H2
PGI_2	Prostacyclin
PI3K	phosphatidylinosital-3-kinase
PIGF	placenta growth factor
PKC	protein kinase C
PLC	phospholipase C
PRE	progesterone response element
PTB	phosphotyrosine binding domain
PTK	protein tyrosine kinase
PTPases	protein tyrosine phosphatases
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
s.flt-1	soluble flt-1 (soluble VEGFR1)
SFJ	sapheno -femoral junction
SH2	Src homology 2 domain
SMC	smooth muscle cells
SP-1	specificity protein 1
SPJ	sapheno-popliteal junction
SSV	short saphenous vein
STAT	signal transducer and activator of transcription
TCF/LEF	T cell factor/lymphoid enhancer transcription complex
TIMP-1	tissue inhibitor of metalloproteases
t-PA	tissue plasminogen activator
tyr	Tyrosine
u-PA	urokinase-type plasminogen activator

UTR	untranslated region
VCAM-1	vascular cell adhesion molecule
VECAD	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGF-A	vascular endothelial growth factor - A
VEGFR1	vascular endothelial growth factor 1 (flt-1)
VEGFR2	vascular endothelial growth factor 2 (KDR)
VEGFR3	vascular endothelial growth factor 3 (flt-4)
VHL	von Hippel Lindau
VPF	vascular permeability factor
vSMC	vascular smooth muscle cells
VSS	venous severity score
VVs	varicose veins
WHO	World Health Organisation
Wnt	wingless pathway

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Chapter 1

INTRODUCTION

1.1 PRIMARY VARICOSE VEINS

1.1.1 Definition of varicose veins

Peripheral vascular disease of the lower limb, broadly speaking can be separated into two categories caused either by blockage of the vein from a blood clot (thrombosis) or through inadequate venous drainage (venous insufficiency) (Nicolaides 2000; Beckman 2002). Chronic venous insufficiency (CVI) is a broad term describing incompetence of the deep, superficial and/or perforating veins (Ricotta *et al.,* 1997) and as such is further subdivided according to the anatomical location where the complication presents, as either deep or superficial disease (Beckman 2002). Superficial CVI describes incompetence of the superficial venous system of the lower limb and is referred to clinically as varicose veins (VVs) (Nicolaides 2000; Beckman 2002). Functional characteristics associated with VVs including disturbed blood flow (reflux/stasis), venous hypertension and dilatation (Browse *et al.,* 1999; Labropoulos *et al.,* 1999; Nicolaides 2000; Ruckley *et al.,* 2002) contribute to further complications including lipodermatosclerosis, haemorrhage and ulceration in a proportion of patients (Shami *et al.,* 1992; Lees *et al.,* 1993; Evans *et al.,* 1999; London *et al.,* 2000). Classically, VVs arising as a result of an identified problem such as deep vein thrombosis are referred to as secondary VVs (Golledge *et al.,* 2003). However in the majority of cases there is no identifiable cause for the development of VVs which are then classified as primary VVs (Nicolaides 2000).

Within this setting, to define varicosity has proved difficult due in part to the complex multimodal venous pathophysiology presented by CVI (Ricotta *et al.,* 1997). Furthermore, a lack of consistency in the diagnostic evaluation of CVI and VVs has led to conflicting reports, both in studies of the clinical management of VVs as well

as data obtained from epidemiology studies. Originally VVs were defined by the WHO as "a saccular dilatation of the veins which are often tortuous"; a definition however, which excludes intradermal subcutaneous veins (venectasis) and any tortuous dilated vein secondary to previous thrombophebitis or ateriovenous fistula (Browse *et al.,* 1999). The definition was amended to include any subcutaneous vein that had permanently lost its valvular efficiency and become elongated, tortuous, pouched and thickened (Dodd *et al.,* 1956). In 1978 the Basle study introduced criteria for the classification of VVs based on their location and graded according to severity (Widmer 1978) and being the most comprehensive, was widely used (Allan *et al.,* 2000).

It is only recently that a comprehensive classification system, developed for the evaluation of severity of functional venous disease (venous reflux) by Duplex ultrasonography, has been agreed by an international committee of the American Venous Forum (Porter *et al.,* 1995). The committee produced a consensus document for the classification and grading of chronic venous disease on the basis of clinical manifestation (C), aetiology (E), anatomical distribution of involvement (A), and underlying pathophysiological findings (P), termed the C.E.A.P. classification (Table 1.1). Accordingly, VVs as defined by C.E.A.P are "palpable subcutaneous, permanently dilated veins equal to or more than 4mm in diameter in the upright position" a definition which includes all refluxing tubular veins including truncal varicosities, tributaries and non-saphenous veins (Porter *et al.,* 1995; Allegra *et al.,* 2003). More recently, the Venous Severity Scoring system was introduced to modify the C.E.A.P. assessment and account for the dynamic changes in outcome with therapeutic management (Rutherford *et al.,* 2000).

CEAP		Classification	Description
Clinical	C0	no visible disease	
	C1	telangiectase or reticular veins	(intradermal venules < 1mm) (subdermal veins to 4mm)
	C2	varicose veins	(subcutaneous, palpable veins >4mm)
	C3	oedema	
	C4	skin changes without ulceration	
	C5	skin changes with healed ulceration	
	C6	skin changes with active ulceration	
A**e**tiology	E_C	congenital disease	
	E_P	primary disease	(undetermined cause)
	E_S	secondary disease	(known pathological)
Anatomy	A_S	superficial veins	1. telangiectase or reticular veins 2. greater saphenous – above knee 3. greater saphenous – below knee 4. lesser (short) saphenous vein 5. nonsaphenous veins
	A_d	deep veins	1. inferior vena cava 2. common iliac 3. internal iliac 4. external iliac 5. Pelvic: gonadal, broad ligament, 6. common femoral 7. deep femoral 8. superficial femoral 9. popliteal 10. crural – anterior/posterior tibial 11. muscular – gastronemial, soleal
	A_P	perforating veins	1. thigh 2. calf
Pathology	P_R	reflux	
	P_O	obstruction	
	$P_{R<O}$	reflux and obstruction	

Table 1.1 C.E.A.P. classification of venous insufficiency (Porter *et al.,* 1995).
The classification was devised to standardize the diagnosis of venous insufficency when considering both anatomic location and pathological features of disease presentation.

1.1.2 Epidemiology of varicose veins

1.1.2.1 Prevalence

VVs are estimated to affect approximately a quarter (10 – 40 %) of the adult population (30 – 70 years) in Westernised societies (Callam 1994; Bradbury *et al.,* 1999; Evans *et al.,* 1999). Approximately 20 percent of patients who present with primary VVs will go on to develop further complications including superficial or deep thrombosis, lipodermatosclerosis, haemorrhage from a superficial varicosity or ulceration (Shami *et al.,* 1992; Lees *et al.,* 1993; Evans *et al.,* 1999; London 2000). Consequently primary VVs and their complications are a cause of considerable morbidity ranking seventh out of the twenty eight chronic diseases listed (Green 1995) and treatment of CVD consumes an estimated 2% of UK's national healthcare resources (Liang 1992).

1.1.2.2 Risk factors

The suggested risk factors for VVs include amongst others gender, age, pregnancy, family history, race, occupation, obesity and diet (Callam 1994; Browse *et al.,* 1999; Evans *et al.,* 1999; Allen *et al.,* 2000; Fowkes *et al.,* 2001; Lee *et al.,* 2003). However, epidemiological data suggests that only family history, age and gender are risk factors associated with an increased incidence of VVs. (Widmer 1978; Abramson *et al.,* 1981; Callam 1994; Fowkes *et al.,* 2001). Most studies suggest women have a higher incidence of VVs (26-38%) than men (10-20%) (Coleridge Smith 1999; Allen *et al.,* 2000) typically presenting with a male to female ratio of 1:3 (1:1.5 to 1:4.4) in the general population (Evans *et al.,* 1999; see Table 1.2).

Study	Female (%)	Male (%)	M:F ratio	Reference
National Health Survey	3.5	0.8	1:4.4	US Dept of Health 1935-36, 1951-61 [1]
UK Survey of Sickness 1950	3.7	2.2	1:1.7	Logan *et al.,* 1957 [1]
Czechoslovakia	14	6.6	1:2.1	Bobek *et al.,* 1966 [1]
Tecumseh Community Health	25.9	12.9	1:2.0	Coon *et al.,* 1973
Basle Study Switzerland	**3.2**	**5.2**	**1:0.6**	Widmer 1978
Western Jerusalem	29.5	10.4	1:2.8	Abramson *et al.,* 1981
New Zealand Maori Community	40	21.5	1:1.9	Beaglehole 1986
Framingham Study	2.6	1.9	1:1.4	Brand F *et al.,*1988
Aachen District	29	14.5	1:2.0	Liepnitz *et al.,* 1989 [2]
Edinburgh Vein Study	**32**	**40**	**1:0.8**	Evans *et al.,* 1999
Edinburgh Vein Study (superficial system)	15	9	1:1.7	Allan *et al.,* 2000

Table 1.2 Prevalence of varicose veins in the general population. Most studies observed an increased incidence of primary VVs in females compared with males with only two studies contradicting the gender bias (in bold). This was attributed to a higher prevalence of mild (Grade 1) trunk varices in men (Edinburgh Vein Study) and a suggested age bias in the Basle study, due in part to a smaller number of younger women in the study ([1]Browse *et al.,* 1999; [2] Evans *et al.,* 1999).

The importance of other risk factors in the development of varicosity is more difficult to determine, due in part to the different methodologies used in the various epidemiological studies. More recently the Edinburgh Vein Study concluded that no strong and consistent lifestyle risk factor could be associated with the development of VVs. (Fowkes *et al.,* 2001; Lee *et al.,* 2003)

1.1.3 **Pathology of varicose veins**

1.1.3.1 Anatomy of the lower limb

The venous anatomy of the lower limb consists of the superficial and deep (below the muscular facia) venous systems which are connected by the perforating veins (communicating system) (Green 1995). Primary VVs are the most common manifestation of CVI and tend to affect the superficial venous system predominately (Beckman 2002). The primary collecting veins of the superficial system are the short saphenous vein (SSV), the greater saphenous vein (GSV) and their tributaries which serve as a conduit passing blood centrally and eventually to the popliteal and femoral veins of the deep system respectively (Figure 1.1) (Beebe-Dimer *et al.,* 2005). The SSV originates in the lateral foot and passes posteriorly lateral to the Achilles tendon in the lower calf, usually lying directly above the deep fascia in the midline as it reaches the upper calf. Here it perforates the deep fascia in the lower part of the popliteal fossa and joins the popliteal vein of the deep system (Gray 1918). The GSV originates in the medial foot and passes upward anterior to medial malleolus crossing the medial tibia in a posterior direction to ascend in the medial line across the knee. Above the knee it continues anteromedially above the deep fascia to the thigh, where it passes through the foramen ovale and joins the common femoral vein at the groin crease (Gray 1918).

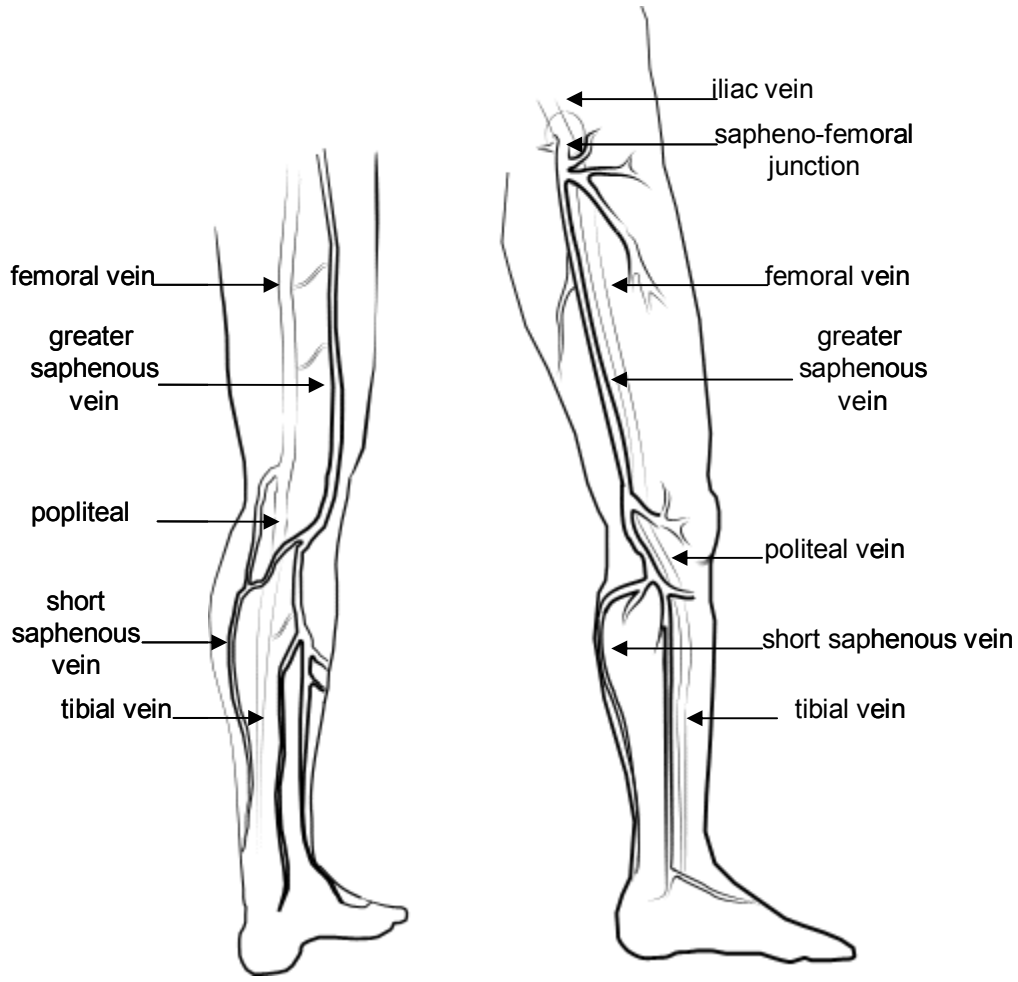


Figure 1.1 The venous anatomy of the lower limb.

Blood from the superficial system (dark) empties into the deep system (light) and is returned to the inferior vena cava via the femoral vein.

The correct venous drainage is from the superficial to the deep systems (Green 1995). Venous blood is pumped out of the deep system of the lower extremities through the action of the plantar venous plexus (foot pump) and the soleal sinuses (calf muscle pump) back to the right atrium of the heart (Araki *et al.,* 1994; Marieb 1998). Blood flow is maintained in a distal to proximal direction in the superficial, deep and perforating veins by bicuspid valves, located along the length of the vein and at positions where the superficial system joins the deep system (Figure 1.2; Ricotta *et al.,* 1997; Marieb 1998). The valves prevent retrograde flow of blood from the deep system back into the superficial system and maintain the pressure differential between the deep system (high pressure) and the superficial systems (low pressure) (Ricotta *et al.,* 1997; Marieb 1998; Beebe-Dimmer *et al.,* 2005).

Historically varicosity was thought to start at points of primary valvular incompetence where the superficial veins communicate with the deep veins, particularly at the SFJ, saphenopopliteal junction (SPJ) and perforating veins (Nicolaides 2000; Golledge *et al.,* 2003). Valvular incompetence allows retrograde flow from the deep system back to the superficial system transferring the normal high pressure of the deep system to the superficial venous system with associated venous hypertension (Green 1995; Nicolaides 2000). Venous hypertension and dilation initiates incompetence of adjacent valves leading to the clinical manifestations of venous reflux and morphological changes of the vessel wall seen with VVs (Nicolaides 2000; Golledge *et al.,* 2003).

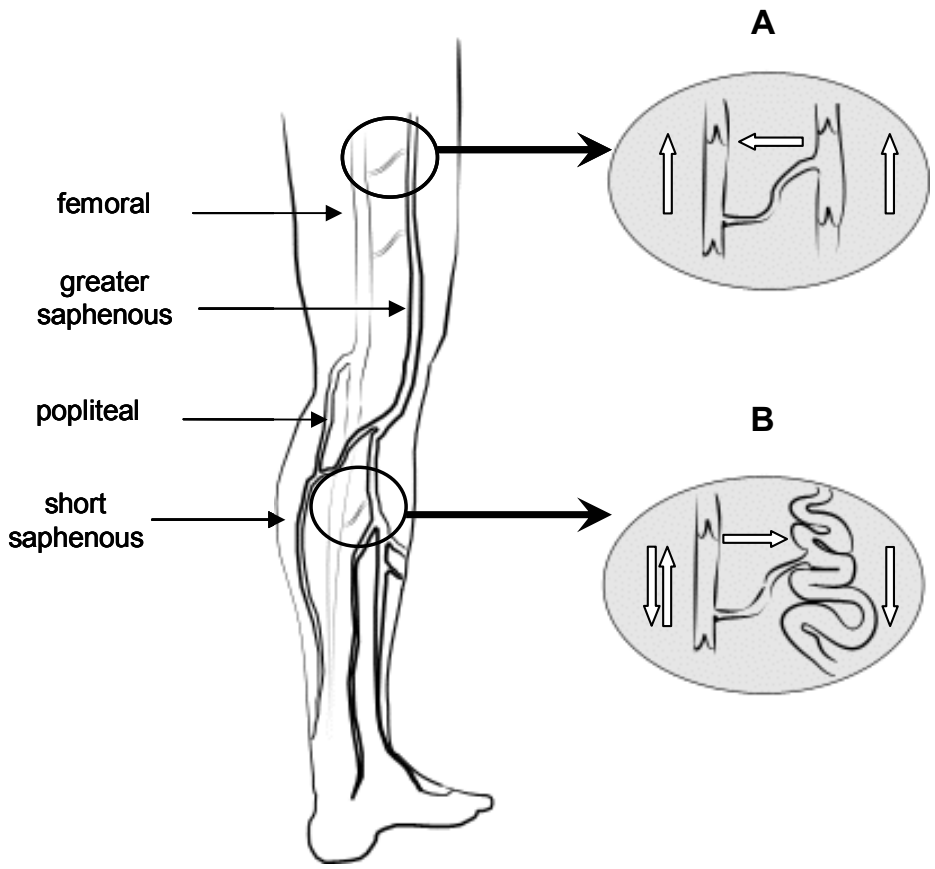


Figure 1.2 Compartmentalisation of the deep and superficial veins in the limb.
Blood from the superficial system (low pressure 20-80mmHg) drains to the deep system (high pressure ~80+ mmHg), the direction of blood flow being maintained in part by competent perforators and venous valves (**A**). Perforator incompetence facilitates the retrograde flow of blood from the deep to superficial system, facilitating the transfer of pressure and blood reflux implicated with vessel wall remodelling in the development of primary VVs (**B**).

1.1.4 **Theories on the development of varicose veins**

Varicosity is a complex pathology characterised by venous hypertension, blood turbulence (stagnation and/or reflux), vascular dilatation and aberrant vessel wall remodelling (Browse *et al.,* 1999; Badier-Commander *et al.,* 2000; Golledge *et al.,* 2003). Several theories describing the development of primary VVs have been proposed, although the precise aetiology remains undefined. Generally however, valvular incompetence following vessel wall dilation is considered the principal determinant of varicosity (Browse *et al.,* 1999), due to either increased venous pressure and/or structural changes to the vessel wall (Golledge *et al.,* 2003; Beebe-Dimer *et al.,* 2005).

Venous pressure and descending valvular incompetence**:** Trendelenburg introduced the concept of descending valvular incompetence as the primary cause of VVs (referenced in Browse *et al.,* 1999). He suggested that venous valves protect the vessel wall below the valve from the pressure in the vein above it. Failure of the SFJ permitted reflux into the GSV which resulted in descending sequential valvular incompetence due to the increased pressure on these sites (Golledge *et al.,* 2003). However, studies of venous valves have demonstrated that the valve can tolerate higher pressure than that of the vein wall itself and that the vessel wall would most probably dilate before the valve became incompetent (Browse *et al.,* 1999). Furthermore a primary defect in the valve cannot explain the histological evidence of apparently normal vein segments in continuity with varicose segments (Badier-Commander *et al.,* 2001) when the whole vein wall is subjected to the same increased pressure (Browse *et al.,* 1999). Finally, more recently the identification of isolated valvular incompetence and reflux at sites distal to a competent SFJ has all contributed

to the evidence contradicting this theory (Abu-Owen *et al.,* 1994; Labropoulos *et al.,* 1999; Labropoulos *et al.,* 2000; Hollingsworth *et al.,* 2001B).

Perforator incompetence: In contrast, incompetence of one or more perforator veins in the lower leg results in retrograde blood flow and elevated pressure from deep to superficial veins during muscular contraction (Figure 1.2) (Nicolaides 2000). Over time, the superficial veins become chronically dilated preventing their apposition, which reverses blood flow. As other perforator valves become incompetent, reflux occurs at additional sites along the GSV eventually initiating incompetence at the SFJ (Nicolaides 2000). This theory can explain why varicosities are often found below competent valves, but not why veins used as arterial bypass conduits (at arterial pressures) become hypertrophic rather than dilated or grossly varicose (Rose *et al.,* 1986; Canham *et al.,* 1997). Furthermore, although trauma induced arteriovenous fistulas cause veins to dilate aberrantly, this returns to normal when the fistulas are repaired, providing further evidence that VVs may not necessarily be caused by elevated pressures unless the walls are inherently weak (Nicolaides 2000).

Structural weakness/dysfunction of the vessel wall: An inherent weakness in the vein wall, due to structural problems in the vessel wall itself, has been proposed to lead to incompetence of the valves when the vessel dilates which results in reflux and development of varicosity (Golledge *et al.,* 2003). Evidence for this theory has come from numerous studies which demonstrate that VVs have a number of abnormalities in their structural components when compared to normal veins. VVs have abnormal collagen and elastin content (increased as well as decreased) (Travers *et al.,* 1992; Gandhi *et al.,* 1993; Venturi *et al.,* 1996), aberrant intimal and medial smooth muscle

cell content (Thulesius *et al.,* 1974; Travers *et al.,* 1992) and different ratios of matrix degrading enzymes when compared to normal veins (Badier-Commander *et al.,* 2000). Furthermore histological analysis of VVs has determined that structural changes in the wall are not distributed uniformly along the vein and segments of gross varicosity are interspersed by seemly normal or atrophic segments (Badier-Commander *et al.,* 2001). A congenital weakness of the wall may be a factor, as more than half of patients affected have a positive family history of VVs (Travers *et al.,* 1996). Primary wall weakness may explain why varicosities are often found below competent valves, but the lack of consistent results from different studies has failed to present a cohesive mechanism for this theory. This may relate to the fact that many of the findings reported are describing final stages of disease and are potentially the result of varicosity and not necessarily the cause (Golledge *et al.,* 2003).

Deregulated venous tone: A further theory is that dilatation results from an inability of the vein to contract adequately, as a result of smooth muscle cell or endothelial dysfunction (Schuller-Petrovic *et al.,* 1997; Brunner *et al.,* 2001; Lowell *et al.*, 1992). Support for this theory has come from numerous investigations that demonstrate abnormal responses of VVs and their components to substances including phenylephrine (Brunner *et al.,* 2001), cyclic GMP (NO) and release of prostacyclin (Schuller-Petrovic *et al.,* 1997) and noradrenalin (Crotty 2003) implicated in the regulation of vascular homeostasis. Notably, these studies suggest a spectrum of disease may exist with dysfunction of the vessel wall being potentially both progressive and associating with more severe disease (Schuller-Petrovic *et al.,* 1997; Brunner *et al.,* 2001).

1.1.5 **Structure of the vein wall**

The vein wall, like that of the artery is composed of three distinct layers, the tunica intima, tunica media and tunica adventitia which surround the vessel lumen (Figure 1.3) (Browse *et al.,* 1999). The muscle layers of the vein, however tend to be thinner and the lumen proportionately larger than those of arteries of a similar size (Marieb 1998). Vein also contains a smaller proportion of elastic tissue in their wall, which allows veins to distend rapidly when filling with blood with minimal change in pressure (Marieb 1998; Ricotta *et al.,* 1997). Indeed, besides being a conduit to return blood to the heart, veins function as blood reservoirs (capacitance vessels) capable of accommodating up to 65% of the blood supply of the body at any given time (Marieb 1998; Green 1995). It is now well established that the cellular components of the vessel wall actively participate in the maintenance of normal blood flow and the regulation of vascular homeostasis (Davies *et al.,* 1993; Gocke *et al.,* 1998; Browse *et al.,* 1999).

1.1.5.1 Tunica intima

The tunica intima is comprised of the vascular endothelium and subendothelium layer, which consists of connective tissue and smooth muscle cells (SMC). The endothelium is a confluent monolayer of simple, squamous endothelium that lines the luminal surface of all blood vessels (Davies *et al.,* 1993; Marieb 1998). Positioned at the interface between the circulating blood and the body's tissue, the endothelium forms a semi-permeable surface that limits passive transfer of cellular and fluid elements between the circulating blood and the tissue (Makrides *et al.,* 1998; Dejana *et al.,* 2004).

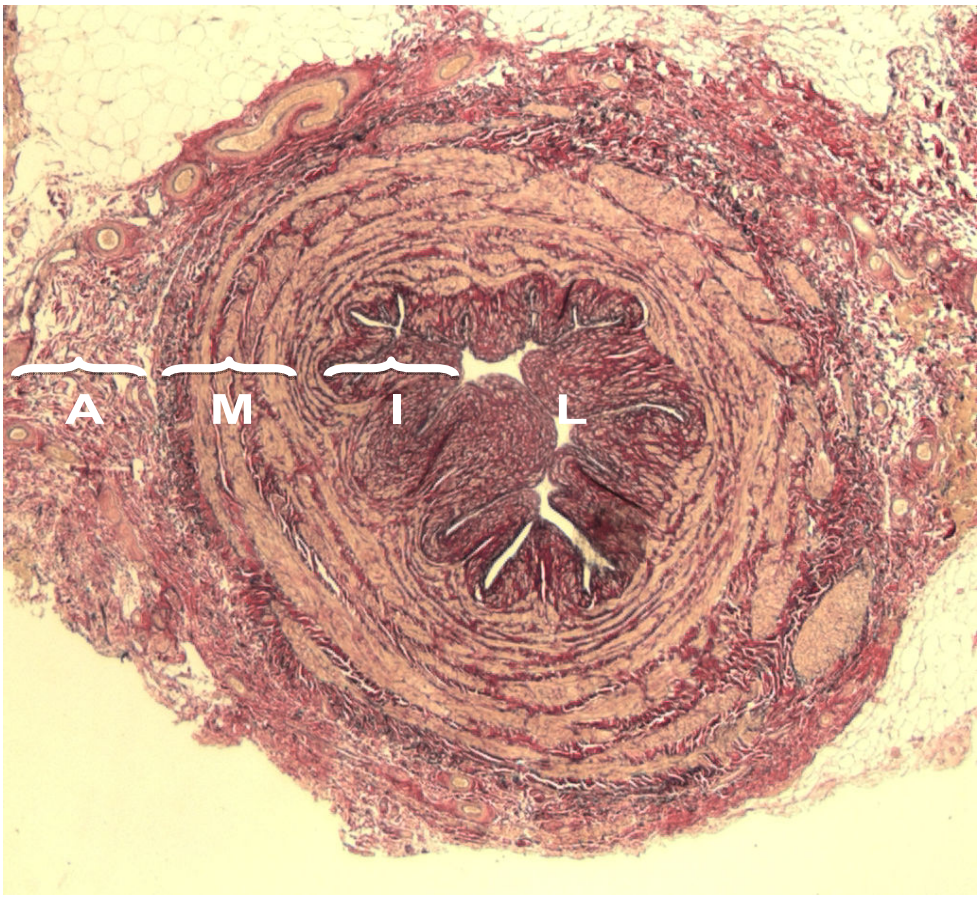


Figure 1.3 Transverse section of the saphenous vein wall (elastic van Gieson-stain). The vein wall is composed of three distinct layers, the tunica adventitia (**A**), tunica media (**M**) and tunica intima (**I**) containing the endothelial cell lining surrounding the vessel lumen (**L**). The majority of the smooth muscle responsible for passive tone is contained in two concentric layers in the tunica media (Woodside *et al.,* 2003). The tunica adventitia, a loosely bound matrix of fibroblasts, smooth muscle cells and extra cellular matrix supports the nerves supplying the sympathetic innervation of the media layer, as well as the vessel's blood supply via the vasa vasorum (Marieb 1998). In addition to bicuspid valves, vein differs to artery by having less muscle and a proportionately larger lumen than artery of a similar size (Browse *et al.,* 1999) (Section published with permission, courtesy Dr M Dashwood, Dashwood *et. al.* 2009).

This strategic location of the endothelium allows it to "sense" changes in circulating blood factors and haemodynamic forces, to which it responds by producing and releasing vasoactive substances (Table 1.3) (Cohen 1998). These agents participate in multiple physiological processes in the vessel wall including the regulation of vascular tone (Cohen 1998; Verma *et al.*, 2002) and blood pressure (Davies *et al.,* 1993), activation of platelets (Gokce *et al.,* 1998), influence blood thrombogenicity and coagulation (Gertler *et al.,* 1992), regulate leukocyte adherence (Gokce *et al.,* 1998) and over the longer term participate in cellular proliferation and matrix degradation typical of vessel wall remodelling. (Quyyumi 1998). Among the more important functions of the endothelium is the integration of the numerous paracine and auotocrine signalling pathways required to regulate vascular homeostasis (Davies *et al.,* 1993; Verma *et al.*, 2002). Consequently, the vascular endothelium participates in the metabolism and transport of a multitude of blood-borne substances to the sub-endothelial tissue (Davies *et al.,* 1993; Makrides *et al.,* 1998; Gokce *et al.,* 1998). In addition, the normal activity of the endothelium maintains a frictionless, anti-thrombotic surface of the lumen by favouring factors that inhibit coagulation and activate fibrinolysis over those that promote thrombosis and endothelial activation (Davies *et al.,* 1993; Keaney 2000; Verma *et al.,* 2002).

Endothelial cells in larger vessels are attached to the surface of the vessel through interaction with the underlying sub-endothelium, a highly organised matrix including collagen, elastin, fibronectin, laminin thrombospondin and glycosoaminoglycans (Davies *et al.,* 1993; Browse *et al.,* 1999). The sub-endothelium provides structural integrity, mechanical strength and elasticity to the vessel wall (Davies *et al.,* 1993). Although endothelial cells secrete a variety of proteases including metalloproteases,

Function	Role	Mediated by
vasomotor tone	dilation	nitric oxide, prostacyclin (PGI_2) bradykinin endothelial derived hyperpolarising factor
	constriction	endothelin, prostaglandin H_2 (PGH_2) endothelium derived constricting factor angiotensin II, platelet derived growth factors reactive oxygen species (superoxide) adenosine triphosphate (ATP) thromboxane A_2
fibrinolysis	anti-fibrinolytic	plasminogen activator inhibitor-1 (PAI-1)
	pro-fibrinolytic	tissue plasminogen activator (t-PA) urokinase-type plasminogen activator (u-PA)
thrombosis	anti-coagulants	heparan sulphate dermatan sulphate thrombomodulin
	pro-coagulants	tissue factor von Willebrand factor
	platelet inhibitors	nitric oxide, prostacyclin (PGI_2)
cell growth	inhibition	nitric oxide heparan sulphate prostacyclin (PGI_2)
	promotion	platelet derived growth factor (PDGF) fibroblast growth factor (bFGF) vascular endothelial growth factor angiotensin II endothelin
matrix degradation	inhibition	tissue inhibitor of metalloproteases (TIMP-1)
	promotion	matrix metalloproteases (MMP-1, 2, 9, 12)
inflammation	anti-inflammatory	nitric oxide
	pro-inflammatory	E-selectin intercellular adhesion molecule (ICAM-1) vascular cell adhesion molecule (VCAM-1) monocyte chemotactic protein (MCP-1) interleukin -8 (IL-8)

Table 1.3 Role of the vascular endothelium in the regulation of homeostasis. (from Gokce *et al.,* 1998)

collagenases, elastases and gelatinases the turnover of the subendothelium in a normal vessel is usually low, being balanced in part by the secretion of inhibitors of the above mentioned proteases (Davies *et al.,* 1993; Browse *et al.,* 1999). Modulation of the connective tissue matrix by synthesis and degradation of its components allows the endothelium to control the activity of the smooth muscle cells and the structure of the vessel wall (Davies *et al.,* 1993; Quyyumi 1998).

1.1.5.2 Tunica media

The tunica media contains the bulk of the smooth muscle of the vein, arranged both circumferentially and longitudinally in the vessel wall interspersed with bundles of connective tissue including collagen and elastic lamina (Marieb 1998; Browse *et al.,* 1999). The size of the tunica media varies considerably in different veins from no smooth muscle cells (small venules, inferior and superior vena carva) to thick layers of smooth muscle fibres interspersed with elastic fibres and collagen (GSV) (Browse *et al.,* 1999).

The media of the GSV is composed of two distinct concentric muscle layers as determined by histology (Badier-Commander *et al.,* 2001). The inner layer appears as thin, clustered longitudinal muscle bundles, while the large outer layer contains circular bundles of SMC arranged in concentric lamellae surrounded by connective tissue (Badier-Commander *et al.,* 2001; Woodside *et al.,* 2003). When examined by electron microscopy, the SMC's appear as spindle shaped cells suggestive of a contractile phenotype (Pappas *et al.,* 1998) and lie in close proximity to each other in parallel arrays surrounded by bundles of regularly arranged collagen fibres (Rose

1986). The elastic network consists of a thin and continuous internal elastic lamina and regular longitudinal medial elastic fibres (Badier-Commander *et al.,* 2001).

The active and variable tone of the vein is regulated by the smooth muscle in the media. The passive tone is provided by the elastic properties of the vein wall which in the resting state is the major source of venous tone (Browse *et al.,* 1999). Changes in tone are mediated through the sympathetic nervous system (adrenergic innervation through nerve endings that terminate in the media) (Browse *et al.,* 1999), local metabolites and circulating or endothelial derived vasoactive substances (Table 1.3; Davies *et al.,* 1993).

1.1.5.3 Tunica adventitia

The tunica adventitia of the GSV is composed of extracellular matrix (collagen and elastin fibres, Elsharawy, *et al.,* 2007) fibroblasts (Satore *et al.,* 2001), and a loosely organized outer longitudinal layer of smooth muscle cells (Canham *et al.,* 1997, Pappas *et al.,* 1998). Historically, the tunica adventitia (and surrounding perivascular tissue) was considered to be merely a supportive layer, thought to protect and anchor the vessel to the surrounding tissue structure (Rose *et al.,* 1986). The nerves responsible for the sympathetic innervation of the tunica media are localised here (Crotty, 2003), as well as the vasa vasorum (or vasa venarum in the vein) which supply the outer muscle layers with oxygen and nutrition (Crotty, 2003; Marieb 1998).

It is now realised that the tunica adventitia is much more than an inert support and the interaction between the tunica adventitia and underlying tunica media and intima

layers is integral to both the survival and proper functioning of the vessel (Crotty, 2003, Dashwood *et al.,* 2004; Siow *et al.,* 2007.). The GSV has an extensive network of vasa venarum which extend deep into the tunica media and in certain cases penetrate the tunica intima (Dashwood *et al.,* 2004; Scotland *et al.,* 2000). Large vasa venarum have a significant SMC content in the vessel wall, while smaller vasa venarum like those typically observe in the tunica media have none (Dashwood *et al.,* 2004) and tend to be thin walled endothelial cell channels (Dashwood *et al.,* 2004).

While the presence of smooth muscle cells suggests the vasa venarum regulate their own tone (Crotty, 2003), they are themselves thought to be actively involved in influencing the tone of the GSV (Dashwood *et al.,* 2004; Scotland *et al*., 2000). The vasa venarum are sensitive to a large number of mediators of vascular tone (Scotland *et al*., 2000) and a considerable portion of the saphenous vessels endothelial (and neuronal) NO is suggested to originate from here (Dashwood *et al.,* 2009).

More direct evidence for the important role of the adventitia is demonstrated from the results of studies into the improved patency of GSV vein grafts (Dashwood *et al.*, 2009). Comparisons of patency rates for vein harvested by conventional techniques, to that of a "no touch" technique (Souza *et al.,* 2009) (which reduces vessel trauma and preserves the structures of the adventitia), highlight the important contribution the adventitia and vascular structures like the vasa venarum have in maintaining saphenous vessel wall reactivity (Souza *et al.,* 2006; Souza *et al.,* 2009).

1.1.6 **Morphology of varicose saphenous vein**

Histological and ultrastructural studies have highlighted significant differences in the composition and organisation of cellular and matrix constituents of varicose vessels compared to normal GSV. Observations from these studies suggest that an overriding feature of varicosity is a loss of organisational structure in the vessel wall as evidenced by the disruption of medial SMC bundles (circular and longitudinal) (Travers *et al.,* 1996), changes in SMC phenotype, fragmentation of elastic lamallae, and inappropriate deposits of extra-cellular matrix components (ECM) like collagen, in different layers of the vessel wall (Rose 1986; Venturi *et al.,* 1996; Badier-Commader *et al.,* 2001; Woodside *et al.,* 2003).

Varicose GSV generally demonstrate an increase in diameter of the vessel lumen and either a gain or loss, in cross sectional area of intima, media and whole wall (Travers *et al.,* 1996; Jones *et al.,* 1999; Badier-Commader *et al.,* 2001; Wali *et al.,* 2002A). This is suggested to occur due to increased transmural pressure and altered vessel wall remodelling (Travers *et al.,* 1996). A large, diffuse thickened intima with intimal hypertrophy and/or hyperplasia and deposits of extra-cellular fibrous tissue is frequently observed (Badier-Commader *et al.,* 2001; Wali *et al.,* 2002A). With intimal hyperplasia, medial SMC transform from a contractile (spindle shape) to a migratory/secretory phenotype (elliptical morphology with numerous vacuoles in the cytoplasm) and migrate from the media to the intima (Pappas *et al.,* 1998; Aguilera *et al.,* 2003). These medial derived SMC release ECM degrading enzymes (Badier-Commader *et al.,* 2000; Woodside *et al.,* 2003; Aguilera *et al.,* 2003) which contribute in part to the degeneration of the basement membrane and fragmentation

of the elastic lamina seen in VVs (Badier-Commader *et al.,* 2000; Woodside *et al.,* 2003; Aguilera *et al.,* 2003).

The media of varicose GSV exhibits large heterogeneity in its thickness due to alternating segments of atrophic and hypertrophic segments (Badier-Commader *et al.,* 2001). Atrophic segments tend to have diminished quantities of SMC and ECM, while hypertrophic segments have relatively more, but more importantly the organisation of the media layer is radically disturbed (Badier-Commader *et al.,* 2001; Wali *et al.,* 2002B). The medial SMC bundles are generally disorganised having lost the parallel array structure inherent with circular or longitudinal orientation (Pappas *et al.,* 1998; Badier-Commader *et al.,* 2001). The structure of the medial SMC layer tends to be interspersed with accumulated fibrous tissue and collagen deposits which totally disrupt the regular pattern of smooth muscle cell bundles (Rose 1986; Venturi *et al.,* 1996). In both atrophic and hypotrophic varicose segments the medial elastic network is highly disorganised and fragmented (Badier-Commader *et al.,* 2001; Wali *et al.,* 2002B).

The pattern of appearance of VVs suggests a progressive disease (Jones *et al.,* 1999; Brunner *et al.,* 2000; Labropoulos *et al.*, 2005) associated with extensive vessel wall remodelling (Gandhi *et al.*, 1993; Venturi *et al.,* 1996; Badier-Commader *et al.,* 2000) which over time results in the dilated, elongated and tortuous veins observed (Travers *et al.,* 1996; Woodside *et al.,* 2003). The structural abnormalities described above, together with their irregular distribution along the vessel (Badier-Commader *et al.,* 2001) and presence of localised dilatation in segments of the vein wall in VVs

(Labropoulos *et al.,* 1999) may suggest that a localised fault (possibly blood borne) potentially could be implicated in the development of VVs.

Here, clear candidates for an early or initiating problem may be with the endothelial cell and/or the smooth muscle cell of the vessel wall (Davies *et al.,* 1993; Gokce *et al.,* 1998; Bundy *et al.,* 2000) where dysfunction of the endothelium may result in reduced smooth muscle action (Lowell *et al.*, 1992). As many of the changes observed in the varicose vessel wall appear to be secondary to the action of the same cytokines required for the regulation of normal vascular homeostasis (Gale *et al.,* 1999; Yancopoulos *et al.,* 2000; Badier-Commader *et al.,* 2001), an imbalance between these mediators may be involved (Schuller-Petrovic *et al.,* 1997, Brunner *et al.,* 2001).

Vascular endothelial growth factor (VEGF) plays a central role in maintaining vascular integrity and reactivity by (in concert with nitric oxide) (Dulak *et al.,* 2003) mediating vaso-permeability and dilatory responses (Ferrara *et al.,* 2003). Previously however, patients with primary VVs, in contrast to normal controls demonstrated a loss of the release of VEGF following a mild, experimentally-induced venous stasis (Hollingsworth *et al.,* 2001A). Furthermore, plasma nitric oxide was reduced significantly suggesting perhaps that a fault in VEGF maintenance of vascular reactivity might be involved (Hollingsworth *et al.,* 2001A).

1.2 VASCULAR ENDOTHELIAL GROWTH FACTOR

In 1983 Senger *et al.* described the partial purification of a novel protein from tumour ascites fluid that induced vascular leakage in the skin of guinea pigs. The protein was named vascular permeability factor (VPF) and was considered to be a specific regulator of tumour blood vessel permeability (Senger *et al.,* 1983). In 1989 two groups independently reported the purification and partial amino acid sequence of an endothelial specific mitogen, purified from pituitary folliculostellate and ATt20 cells respectively, which they named vascular endothelial growth factor (VEGF) and vasculotropin respectively (Ferrara *et al.,* 1989; Ploüet *et al.,* 1989). Molecular cDNA cloning and characterisation of protein by sequencing demonstrated that VPF and VEGF were encoded by the same gene and were indeed the same molecule (Leung *et al.,* 1989; Keck *et al.,* 1989, Senger *et al.,* 1990). Furthermore, it was demonstrated that VEGF was a potent and specific mitogen for vascular endothelial cells and that several VEGF isoforms are produced from this gene by alternative splicing to form active disulphide linked homodimers (Ferrara *et al.,* 1989; Leung *et al.,* 1989; Tischer *et al.,* 1991; Keck *et al.,* 1989). More importantly, this was the first demonstration that VEGF could have multiple regulatory functions on blood vessel physiology, including the stimulation of endothelial proliferation and migration, as well as the regulation of endothelial monolayer permeability (Senger *et al.,* 1983; Ferrara *et al.,* 1989; Ploüet *et al.,* 1989; Keck *et al.*, 1989).

Several additional members of the VEGF family have been identified based on their structural homology to the original VEGF (VEGF-A). They include four genes for *VEGF* (*VEGF B-E*) and a related growth factor, placenta growth factor *(PIGF)*, all of which contain eight conserved cysteine residues required for dimerisation and

binding of the ligand to the respective VEGF receptors (Klagsbrun *et al.,* 1996). VEGF-A shares approximately 20% amino acid homology with platelet derived growth factor (PDGF), while PIGF shares 53% amino acid sequence homology to the PDGF-like region of VEGF-A (Keck *et al.,* 1989; Maglione *et al.,* 1991). PIGF expression is restricted to the placenta where alternative splicing generates three distinct isoforms (Maglione *et al.,* 1991). VEGF-A/PIGF heterodimers exist *in vivo* but have weaker mitogenic activity than VEGF-A homodimers and might represent a mechanism for modulating VEGF-A bioactivity (Cao *et al.,* 1996). VEGF-B has 43% homology to VEGF-A_{165} and is expressed in a variety of tissues but is particularly abundant in the heart and skeletal muscle (Olofsson *et al.,* 1996). VEGF-B can form heterodimers with VEGF-A which, when expressed in the same cell remain associated with the cell surface and perhaps indirectly control VEGF-A release and bioavailability (Olofsson *et al.,* 1996). The 23kDa VEGF-C is a potent mitogen for endothelial cells *in vitro* (Joukov *et al.,* 1996). VEGF-C has 30% homology to VEGF-A_{165}, but is more closely related to VEGF-D (Joukov *et al.,* 1996). VEGF-C is thought to participate in the development of both embryonic vascular and lymphatic tissue, but in adult tissue probably acts as a paracine regulator of lymphanogenesis and maintenance of lymphatic vessels (Jeltsch *et al.,* 1997; Joukov *et al.*, 1997; Kukk *et al.*, 1996). Less is known of VEGF-D (c-fos induced growth factor), but expression patterns in mice suggest an important role in the development of lung tissue (Farnebo *et al.,* 1999). All of these polypeptides are endogenously expressed in mammals, except for VEGF-E which is encoded by a double stranded DNA parapoxvirus, *orf* and can induce microvascular proliferation and dilation (Meyer *et al.,* 1999; Wise *et al.,* 1999).

Following the discovery of VEGF, the specific receptors for VEGF-A were identified through binding studies and the genes encoding the tyrosine kinase receptors were isolated (Vaisman N *et al.,* 1990; Devries *et al.,* 1992; Terman *et al.,* 1992; Millauer *et al.,* 1993). It is now realised that VEGF family of homodimeric glycoproteins mediate their effect through interaction with three signalling tyrosine-kinase receptors: VEGFR1 (flt-1, *fms* like kinase 1) including a soluble truncated form, soluble flt-1 (s.flt-1), VEGFR2 (KDR, kinase domain receptor, flk1) and VEGFR3 (flt-4) in addition to an accessory receptor (neuropilin) (Ferrara *et al.,* 2003). The various members of the VEGF family have overlapping affinities to the receptors with VEGFR2 binding VEGF-A, C and D, whereas VEGFR1 binds VEGF-A & B and PIGF specifically, while VEGFR3 binds VEGF-C and VEGF-D only (Neufeld *et al.,* 1999; Ferrara 1999; Yancopoulos *et al.,* 2000; Figure 1.4).

VEGFR1 and VEGFR2 confer the endothelial specificity of VEGF-A as they are expressed predominantly in endothelial cells (Millauer *et al.,* 1993; Yamaguchi *et al.,* 1993; Quinn *et al.,* 1993; Fong *et al.,* 1995). A few additional cell types express one or both of these receptors, as VEGFR1 expression has been demonstrated in trophoblast cells, monocytes (Clauss *et al.*, 1996; Barleon *et al.,* 1996) and renal mesangial cells, while VEGFR2 is also expressed in haematopoietic stem cells, megakaryocytes and retinal progenitor cells (Neufeld *et al.,* 1999). The expression of VEGFR3 becomes restricted mainly to the lymphatic endothelium of adult tissue (Pajusola *et al.,* 1992) where it is suggested to have a role in the regulation of lymphangiogenesis (Kukk *et al.,* 1996; Jeltsch *et al.,* 1997). The co-receptor neuropilin, demonstrated to play a role in axon guidance is implicated in VEGF-A_{165} induced migration of EC in blood vessel development (Soker *et al.,* 1998).

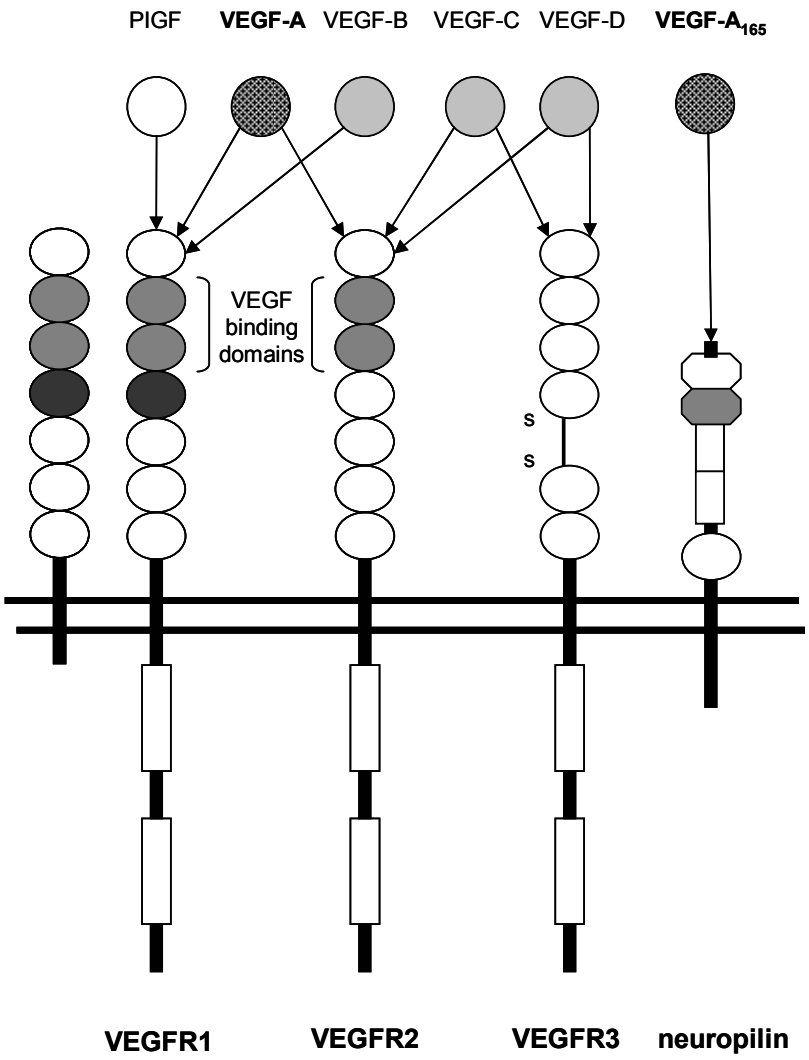


Figure 1.4 Interaction of VEGF protein family with VEGF receptors.
Arrows indicate the documented interaction of VEGF family members with each specific receptor. VEGFR1 selectively binds PIGF, VEGF-A and VEGF-B, while VEGFR2 binds VEGF-A, C and D. VEGFR3 is thought to be involved in lymphangiogenesis and is a selective receptor for VEGF-C and D specifically. Neuropilin binds VEGF-A_{165} only (adapted from Yancopoulos *et al.,* 2000).

1.2.1 **Molecular characteristics of *VEGF-A***

The *VEGF-A* gene is localised on chromosome 6p21, spans approximately 14kb, and is comprised of eight exons separated by seven introns (Tischer *et al.,* 1989). To date five isoforms of *VEGF-A* have been identified all the result of alternative splicing (exons 6 and 7) of the mature 3.2kb message RNA (Tischer *et al.,* 1989; Houck *et al.,* 1991; Poltorak *et al.,* 1997). The resulting proteins range in length from 121 to 206 amino acids (VEGF-A_{121} VEGF-A_{145} VEGF-A_{165} VEGF-A_{189} VEGF-A_{206}) and are all capable of inducing EC proliferation, albeit with different efficiency (Figure 1.5) (Keyt *et al.,* 1996A). VEGF-A_{121} and lacks the amino acids encoded by both exons 6 and 7, whereas VEGF-A_{145} lacks exon 7 but is distinguished by the presence of only the first 21 amino acid residues of exon 6 (Tischer *et al.,* 1991; Poltorak *et al.,* 1997). VEGF-A_{165} lacks the 41 amino acid residues encoded by exon 6, while VEGF-A_{189} lacks 17 amino acids encoded by exon 6 (Tischer *et al.,* 1991). VEGF-A_{206} is the only isoform to contain all 8 coding exons (Tischer *et al.,* 1991). The varied properties of different VEGF-A protein isoforms are dependant in part to the specific exons that are spliced, which affect the heparin binding capability, secretory characteristics, affinity to VEGF receptors and mitogenic potency of the isoform (Park *et al.,* 1993; Keyt *et al.,* 1996B).

Although transcripts encoding VEGF-A_{121} VEGF-A_{165} and VEGF-A_{189} are detected in most cell types that express VEGF-A, VEGF-A_{165} is the predominant molecular species produced by a variety of normal and transformed cells (Houck *et al.,* 1991). In contrast the expression of VEGF-A_{206} and VEGF-A_{145} is rare (Houck *et al.,* 1991). Native VEGF-A (corresponding to VEGF-A_{165}) is a homodimeric glycoprotein of

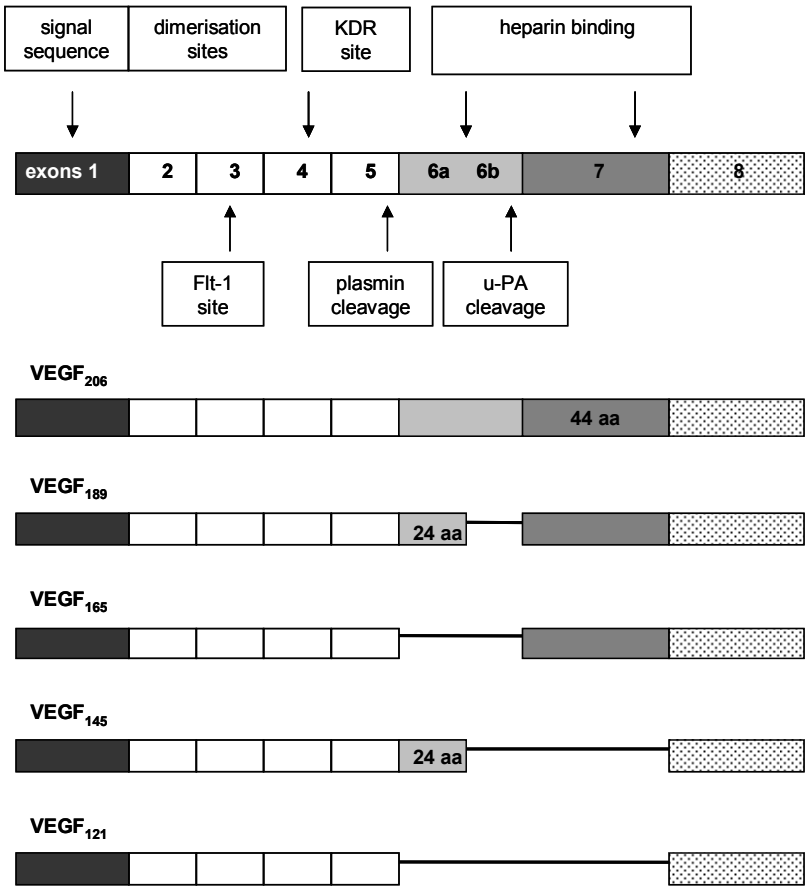


Figure 1.5 Molecular structure of the *VEGF-A* gene.

The various protein isoforms are derived from alternative splicing of the mature VEGF-A message. The mRNA sequence that code for protein domains required for VEGF homodimerisation and binding VEGF receptors and heparin are annotated (adapted from Bates *et al.,* 2002).

45 kDa. VEGF-A_{165} is a basic heparin-binding protein due to the presence of 15 basic amino acids within the 44 residues encoded by exon 7 (Ferrara *et al.,* 1989; Ploüet *et al.,* 1989; Houck *et al.,* 1992). In contrast VEGF-A_{121} which lacks both this region and exon 6 is weakly acidic and does not bind heparin (Houck *et al.,* 1992). Consequently VEGF-A_{121} is freely secreted from VEGF-A producing cells, while VEGF-A_{165} is released, although a significant quantity remains bound to the cell surface and ECM (Park *et al.,* 1993). As VEGF-A_{189} and VEGF-A_{206} contain additional sequences in exon 6 that mediate heparin binding and bind heparin with great affinity, they tend to be sequestered in the ECM (Houck *et al.,* 1992; Park *et al.,* 1993).

VEGF-A_{189} and VEGF-A_{206} can be release from ECM by heparin, plasmin (following cleavage of the COOH terminus) and urokinase type plasminogen activator (uPA) (Houck *et al.,* 1992; Park *et al.,* 1993; Ploüet *et al.,* 1997). Indeed, proteolytic cleavage of recombinant VEGF-A_{189} and VEGF-A_{206} is required to exert their mitogenic effect and uPA-VEGF-A fragments have equivalent endothelial cell mitogenicity to that of VEGF-A_{165} (Houck *et al.,* 1992; Ploüet *et al.,* 1997). Although not all VEGF-A isoforms contain a site for uPA cleavage they all are cleaved by plasmin in exon 5 (Figure 1.5). However, the plasmin VEGF-A fragment (VEGF-A_{110}) elicits a 50 fold reduced mitogenic effect on endothelial cells (similar to that observed for VEGF-A_{121}) compared to VEGF-A_{165} and uPA-VEGF-A_{189} (Keyt *et al.,* 1996B; Ploüet *et al.,* 1997). This suggests that the domains encoded by exon 6 and 7 not only regulate the availability of VEGF-A through heparin sulphate binding but also modulate the activity and mitogenic potency of VEGF-A (Houck *et al.,* 1992; Klagsbrun *et al.,* 1996; Ploüet *et al.,* 1997).

All VEGF-A protein isoforms are secreted as covalently linked homodimers which associate initially through hydrophobic interactions before being stabilised by Cys^{51} to Cys^{61} inter-chain disulphide bonds (Potgens *et al.,* 1994). The signal peptide comprising exon 1 and four residues of exon 2, contains an amphipathic α-helix required for dimerisation which is cleaved off during secretion (Keck *et al.,* 1989; Siemeister *et al.,* 1998). Site directed mutagenesis identified three acidic residues (Asp^{63}, Glu^{64} and Glu^{67}) in exon 3 required for binding of VEGF-A to VEGFR1 (Keyt *et al.,* 1996A). Conversely, exon 4 of VEGF-A contains three basic residues (Arg^{82}, Lys^{84} and His^{86}) essential for binding to VEGFR2 (Keyt *et al.,* 1996A). Three highly flexible loops are clustered at each pole of the dimer interface. Loop II contains the VEGFR1 binding determinants and lies close to loop III of the opposing monomer, which binds to VEGFR2 (Keyt *et al.,* 1996A). The positioning of these receptor-binding interfaces at each pole of the VEGF-A seems to facilitate receptor dimerisation, which is essential for transphosphorylation and signalling as mutant dimers that have only one receptor binding site antagonise native VEGF-A activity (Siemeister *et al.,* 1998).

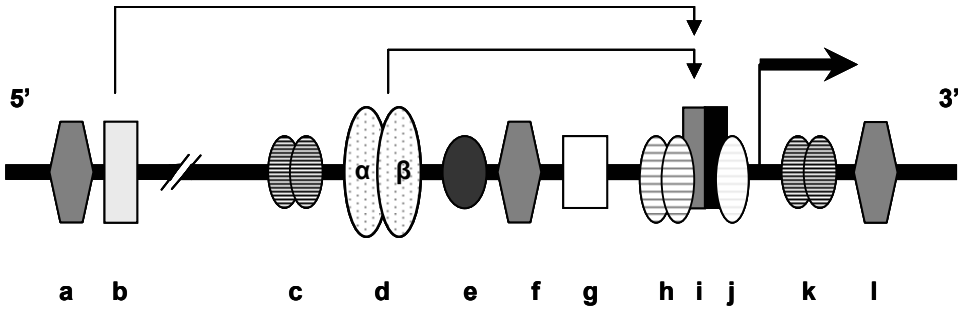
1.2.2 **Regulation of *VEGF-A* expression**

VEGF-A is produced by numerous cell types besides endothelial cells, including vascular smooth muscle cells (Tischer *et al.,* 1991), pericytes, fibroblasts, keratinocytes, macrophages, lymphocytes, megakaryocytes, neutrophils, basophils, mast cells, astrocytes and tumour cells (Neufeld *et al.,* 1999; Ferrara 1999). The generation of VEGF-A mRNA is regulated both at the transcriptional (Tischer *et al.,* 1989; Houck *et al.,* 1992) and post transcriptional level (Levy *et al.,* 1996), while post translational processing is also implicated in regulating VEGF-A protein availability

and activity (Dulak *et al.,* 2003; Lee *et al.,* 2005). Transcriptional regulation of *VEGF-A* synthesis is mediated through the binding of several transcription factors including HIF-1, AP-1 (activator protein-1), AP-2, SP-1, CREB and STAT3 to consensus sequences present in the promoter region of the *VEGF-A* gene (Figure 1.6) (Tischer *et al.,* 1989; Pages *et al.,* 2005).

Hypoxia: Hypoxia induces a rapid and potent increase in *VEGF-A* transcription (other *VEGF* family members are not upregulated by hypoxia) mediated in part by the binding of HIF-1 (heterodimer of HIF-1α and HIF-1β, aryl hydrocarbon receptor nuclear translocator, ARNT) to the 47bp hypoxia response element (HRE) located at -985 to -939bp upstream from the transcriptional start in the *VEGF-A* gene promoter (Liu *et al.,* 1995; Levy *et al.,* 1995). In addition, hypoxia increases the half life of *VEGF-A* mRNA (which is intrinsically labile under normoxic conditions) between three to eight fold (Levy *et al.,* 1996) through the binding of a hypoxia induced stability factor (HuR) to sequences in both the 5' and 3' untranslated region (UTR) (Shima *et al.,* 1995; Levy *et al.,* 1996; Levy *et al.,* 1998). Furthermore, the 5' UTR contains both an alternative transcription initiation site and internal ribosomal entry site (IRES), which facilitates enhanced translation of uncapped *VEGF-A* mRNA via the IRES in conditions of stress (like hypoxia) that inhibits cap-dependant translation (Akiri *et al.,* 1998).

Nitric Oxide: Nitric oxide (NO) induces *VEGF-A* synthesis in numerous cell types, including vascular smooth muscle cells, macrophages, keratinocytes, and tumour cells (Dulak *et al.,* 2003). NO influences *VEGF-A* transcription by a mechanism independent of guanylyl cyclase activation (Kimura *et al.,* 2000), with NO mediated



a. - progesterone response element (PRE) - TGTACA

b. - oestrogen response element (ERE) - AATCAGACTGAC

c. - adenosine phosphate transcription factor 1 (AP 1) - TGAATGCA

d. - hypoxia response element, (HRE) - TACGTGGG

e. - signal transducer and activator of transcription 3 (STAT3) - TTCCCAAA

f. - progesterone response element (PRE) - TGTACA

g. - Liver X receptors α and β (LXR) - TGTCCcacgTAACCT

h. - (SP 1) - GGGCGG / CCGCCC

i. - adenosine phosphate transcription factor 2 (AP 2) - GGCCGGG

j. - (EGR) - CCGGGGC

k. - adenosine phosphate transcription factor 1 (AP 1) - TGAGTGA

l. - progesterone response element (PRE) - TGTTCT

Figure 1.6 Molecular structure of the *VEGF-A* gene promoter.

The promoter region binding sites for transcription factors implicated in the transcriptional activation of *VEGF-A* (adapted from Pages *et al.,* 2004).

transcription of *VEGF-A* being dependant on MAPK and protein kinase B (Akt) activation of transcription factors like AP-1 and hypoxia-inducible factor (HIF-1) (Kimura *et al.,* 2000; Kimura *et al.,* 2003). Furthermore, effects of NO on VEGF-A production are similarly mediated by heme oxygenase (HO-1), an enzyme generating carbon monoxide, iron and biliverdin from its substrate heme, upregulated by hypoxia (Dulak *et al.,* 2003). With respect to NO effects on VEGF-A expression therefore, NO mimics hypoxia by acting as an intracellular activator of HIF-1α (Kimura *et al.,* 2000) the classic activator of *VEGF-A* gene transcription, (Sandau *et al.,* 2001; Dulak *et al.,* 2003).

Cytokines, growth factors and inflammation: A large number of factors both elevate *VEGF-A* gene transcription and/or induce the release of VEGF-A including cytokines (IL-1β, IL-6, TNF-α), growth factors (EGF PDGF-β, bFGF, TGF-β, KGF), prostaglandins (PGE_1, PGE_2, PGJ_2), cyclic AMP (reviewed in Neufeld *et al.,* 1999; Ferrara *et al.,* 2003). In addition, reactive oxygen species (H_2O_2, NO) added exogenously or derived from activated macrophages, neutrophils, vSMC and endothelial cells, upregulate *VEGF-A* gene transcription in various cell and animal models (Neufeld *et al.,* 1999; Ferrara *et al.,* 2003).

Transformation and differentiation: Many tumours secrete high concentrations of VEGF-A and several specific cell transformation events including p53 activating mutations and oncogenic mutations (or amplification) of *ras* are associated with increased *VEGF-A* expression and angiogenesis in developing tumours (Neufeld *et al.,* 1999). The von Hippel-Lindau tumour suppressor gene (VHL) is also implicated in the regulation of *VEGF-A* expression in von Hippel-Lindau disease and sporadic

clear cell renal carcinomas (Ferrerra 1999). VHL is a negative regulator of hypoxia induced transcription of many genes, including the transcription of *VEGF-A* (Siemester *et al.,* 1996). Wild type VHL sequesters protein kinase C isoforms (PKCζ and PKCδ) which prevents their translocation from the cell membrane and activation of the MAPK signalling pathway leading to the induction of *VEGF-A* transcription (Pal *et al.,* 1997). At the level of transcription, VHL forms a complex with the SP-1 transcription factor inhibiting the binding of SP-1 to the *VEGF-A* gene promoter, which prevents SP-1 mediated *VEGF-A* transcription (Tischer *et al.,* 1991). Mutation of VHL therefore causes a loss of suppression of *VEGF-A* transcription in these pathologies (Siemester *et al.,* 1996; Ferrerra 1999).

Post-translational regulation of VEGF-A availability: As stated (1.2.1) the domains encoded by exon 6 and 7 regulate the availability of VEGF-A through heparan sulphate binding, as well as modulating the potency of the released VEGF-A protein (Klagsbrun *et al.,* 1996). As such the binding of VEGF-A to components of the ECM is thought to be a reservoir for the storage of growth factor, used to mediate both the availability and activity of VEGF-A (Houck *et al.,* 1992; Ploüet *et al.,* 1997). Furthermore, this suggests that VEGF-A protein is made available to endothelial cells by two different mechanisms, either directly through secretion by VEGF-A producing cells or following release by proteolytic cleavage from the ECM (Ferrara 1999). Notable, as VEGF-A is itself demonstrated to up-regulate the expression of proteases implicated in matrix degradation (Pepper *et al.,* 1991) in effect it may regulate the release of matrix bound VEGF-A in a positive feedback mechanism.

1.2.3 **Molecular characteristics of VEGF receptors**

In vascular endothelium, VEGF-A's mode of action occurs through the activation of either one or both of the homologous high affinity protein tyrosine kinase receptors (RTK), the 180kDa VEGFR1 (flt-1, *fms* like tyrosine kinase receptor) or 230kDa VEGFR2 (KDR, kinase insert domain receptor) (Vaisman *et al.,* 1990; Devries *et al.,* 1992; Terman *et al.,* 1992; Millauer *et al.,* 1993). The VEGF receptors are characterised by the presence of seven extra-cellular immunoglobulin-like loops, a trans-membrane region and a conserved intracellular tyrosine kinase domain interrupted by a kinase insert sequence (split tyrosine kinase domain) and represent a new family of tyrosine kinase receptors (Figure 1.7) (Shibuya *et al.,* 1990; Terman *et al.,* 1992; Tanaka *et al.*, 1997; Shinkai *et al.,* 1998). More recently, a soluble truncated form of VEGFR1 (s.flt-1) comprised of only the first six immunoglobulin-like domains has been identified and is proposed to function as a specific high–affinity antagonist of VEGF-A function (Kendall *et al.,* 1993; Kondo *et al,* 1998; Belgore *et al.*, 2000).

Besides the two VEGF receptors, VEGF-A_{165} (exon 7 domain) binds the co-receptor neuropilin 1 (NRP1) the neuronal receptor of the semaphorin family (Soker *et al.,* 1996; Soker *et al.,* 1998). NRP1 has a wide tissue distribution including the vascular endothelium, but lacking an intracellular domain is suggested to enhance the binding of VEGF-A_{165} to VEGFR2 and play a role in VEGFR2 dependent migration of endothelial cells in response to VEGF-A_{165} (Soker *et al.,* 1998).

Binding of VEGF-A induces homodimerisation of the VEGF receptors followed by autophosphorylation and activation of downstream signalling cascades (Zachary *et*

al., 2001; Ferrara *et al.,* 2003). VEGFR1 is reported to have a greater affinity for VEGF-A_{165} (*K*d 10-30pM,) than VEGFR2 (75-125pM), although binding studies report a 10 fold increase in the level of expression of VEGFR2 on EC (Devries *et al.,* 1992; Waltenberger *et al.,* 1994). Indeed receptor binding studies investigating phosphorylation status following stimulation with VEGF-A suggest, that VEGFR2 is the major mediator of the mitogenic, angiogenic and permeability enhancing effects of VEGF-A (Terman *et al.,* 1992; Millauer *et al.,* 1993; Waltenberger *et al.,* 1994; Ferrara *et al.,* 2003). Conversely, VEGFR1 undergoes weak tyrosine autophosphorylation in response to VEGF-A in EC and is proposed to be a decoy receptor, negatively regulating the activity of VEGF by both preventing VEGF-A binding to VEGFR2 and modulating VEGFR2 activity (Devries *et al.,* 1992; Park *et al.,* 1994; Waltenberger *et al.,* 1994; Zeng *et al.,* 2001).

Further evidence for this role has come from the identification of a soluble truncated form of VEGFR1, s.flt-1 which inhibits the activity of VEGF-A by sequestering it from VEGFR1 and VEGFR2 (Kendall *et al.,* 1993; Kendall *et al.,* 1996A; Barleon *et al.*, 1997B; Ferrara *et al.,* 1998; Barleon *et al.,* 2001). Furthermore s.flt-1 is proposed to directly modulate the action of VEGFR2 by forming nonsignaling dimers, down-regulating the phosphorylation status of the receptor (Kendall *et al.,* 1993; Kendall *et al.,* 1996A; Graubert *et al.,* 2001). Finally, in mice the absence of VEGFR1 induces embryonic death which can be prevented by the presence of the extra-cellular IgG domains of VEGFR1 which allows normal development (Fong *et al.,* 1995; Hiratsuka *et al.*, 1998).

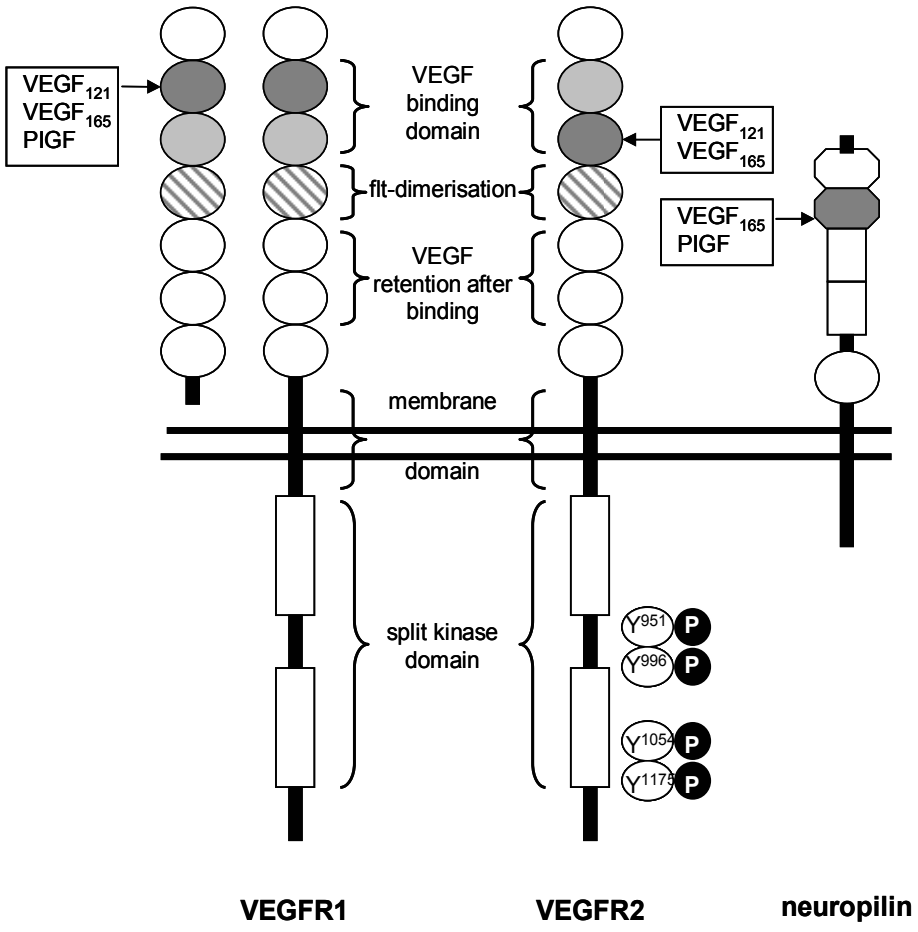


Figure 1.7 Molecular structure of the VEGF-A receptors.

Structure of VEGFR1 (membrane and soluble flt-1), VEGFR2 (KDR) and neuropilin illustrating extra-cellular IgG-like, trans-membrane and intra-cellular split kinase domains. Proposed domain function, VEGF-A binding sites and VEGFR2 major tyrosine phosphorylation sites are annotated (adapted from; Tanaka *et al.*, 1997; Shinkai *et al.*, 1998; Ferarra 1999).

1.2.4 **Regulation of VEGF receptor expression**

The expression of VEGFR1 and VEGFR2 is predominantly restricted to the vascular endothelium (Millauer *et al.,* 1993; Quinn *et al.,* 1993). Both receptors contain control elements in their promoter regions essential for endothelial cell specific transcription (Morishita *et al.,* 1995; Patterson *et al.,* 1995). However, a few additional cell types do express the VEGF receptors, notably the expression of VEGFR1 on monocytes which mediates VEGF-A induced chemotaxis and tissue factor production in these cells (Barleon *et al.,* 1996; Clauss *et al.,* 1996; Neufeld *et al.,* 1999). Generally, in EC the regulation of VEGF receptor expression is coordinated with that of VEGF-A (Wang *et al.,* 2000) as factors that up regulate VEGF-A expression tend to induce receptor expression (Shen *et al.,* 1998).

Hypoxia: *VEGFR1* (membrane and soluble), like *VEGF-A* contains a hypoxia response element (HRE, position -976 to -937) in its promoter and is potently upregulated by hypoxia in a HIF-1 dependant mechanism (Liu *et al.,* 1995; Gerber *et al.,* 1997; Huckle *et al.*, 2004). In contrast, the promoter for *VEGFR2* lacks a HRE and transcription of the receptor is not induced by hypoxia (Gerber *et al.,* 1997; Waltenberger *et al.,* 1996). However the expression of VEGFR2 is increased by hypoxia indirectly at the post transcriptional level, by paracine factors released from ischaemic tissue and hypoxic dependant increases in VEGF-A expression (Brogi *et al.,* 1996; Waltenberger *et al.,* 1996; Wang 2000).

Growth factors: Numerous growth factors affect the expression of the VEGF receptors in endothelial cells including VEGF-A (Barleon *et al.,* 1997A). VEGF-A stimulation of VEGFR2 potently induced both transcription and protein expression

of VEGFR2 in adrenal cortex EC (Shen *et al.,* 1998). Similarly up regulation of VEGF-A is associated with elevated VEGFR1 (both membrane and soluble isoforms) expression in human EC (Barleon *et al.,* 1997A). Conversely, down regulation of VEGFR2 receptor expression and desensitisation of cellular responsiveness following chronic incubation with VEGF-A is coupled with up regulation of *VEGFR2* gene transcription (Wang *et al.,* 2000). Consistently, an increase in VEGF-A expression in EC models results in elevated VEGF receptor expression suggesting that VEGF-A modulates the expression of its own receptors in a positive feedback mechanism (Shen *et al.,* 1998; Wang *et al.,* 2000).

Heparan sulphate proteoglycans: VEGF receptor function is further modulated by heparin and heparan sulphate proteoglycans (HSPGs) (Fairbrother *et al.*, 1998). For VEGFR2, low concentrations of heparin sulphate (0.1-10μg/ml) strongly potentiate the binding of VEGF-A_{165}, while conversely higher concentrations inhibit $VEGF_{165}$ binding (Gitay-Goren *et al.,* 1992). This effect is heparin specific as depletion of cell surface HSPG by heparinase treatment severely reduces binding, which is restored with the addition of exogenous heparin (Terman *et al.,* 1994). As VEGF-A_{121} lacks both exons 6 and 7 (heparin binding sites) binding of VEGF-A_{121} to VEGFR2 is unaffected by heparinase or exogenous heparin (Gitay-Goren *et al.,* 1992). For VEGFR1, even though both VEGF-A_{165} and VEGF-A_{121} binding are inhibited by heparin, the presence of HSPG is required for proper functioning as treatment with heparinase totally abolishes binding to VEGFR1 (Cohan *et al.,* 1995).

1.2.5 **Mechanisms of action and signal transduction**

VEGF-A activates almost all known endothelial cell signalling pathways *in vitro* (Bates *et al.*, 2002) with the identification of over forty specific signalling molecules activated in response to VEGF-A signalling (Zachary *et al.,* 2001). As discussed in chapter 1.2.1. receptor binding studies suggest that in vascular EC, VEGF-A signalling is mediated predominantly via VEGFR2 (Vaisman *et al.*, 1990; Millauer *et al.,* 1993; Waltenberger *et al.,* 1994). Although VEGFR1 is weakly tyrosine phosphorylated in response to VEGF-A (Hiratsuka *et al.*, 1998), in EC its activation is not associated with the mitogenic, angiogenic and permeability enhancing effects of VEGF-A (Millauer *et al.,* 1993; Waltenberger *et al.,* 1994). The discussion below therefore, will concentrate primarily on the signalling mechanism(s) of VEGFR2 in the vascular endothelium.

VEGFR2 signal transduction follows the consensus scheme for activation of receptor tyrosine kinases (RTK) (Schlessinger 2000). Binding of VEGF-A induces receptor dimerisation (Potgens *et al.,* 1994; Fuh *et al.,* 1998) resulting in autophosphorylation of five specific tyrosine residues within the dimeric complex (Figure 1.8) including Tyr^{951}, Tyr^{996} (kinase insert domain); Tyr^{1054}, Tyr^{1059} (kinase domain) and Tyr^{1175} and Tyr^{1214} (C terminal tail) (Vaisman N *et al.,* 1990; Terman *et al.,* 1992; Millauer *et al.,* 1993; Kendall *et al.*, 1999; Matsumoto *et al.,* 2001). Tyr^{1054} and Tyr^{1059} are located in the activation loop of the tyrosine kinase domain and are required for maximal activation of the VEGFR2 kinase (Dougher *et al.*, 1999). Both Tyr^{1059} and the COOH terminal Tyr^{1175} appear to mediate VEGF induced proliferation as mutation of Tyr^{1059} leads to a loss of intracellular Ca^{2+} mobilisation and ERK activation (Zeng *et al.,* 2001) while Tyr^{1175} activates phospholipase C (PLCγ) (Takahashi *et al.,* 2001).

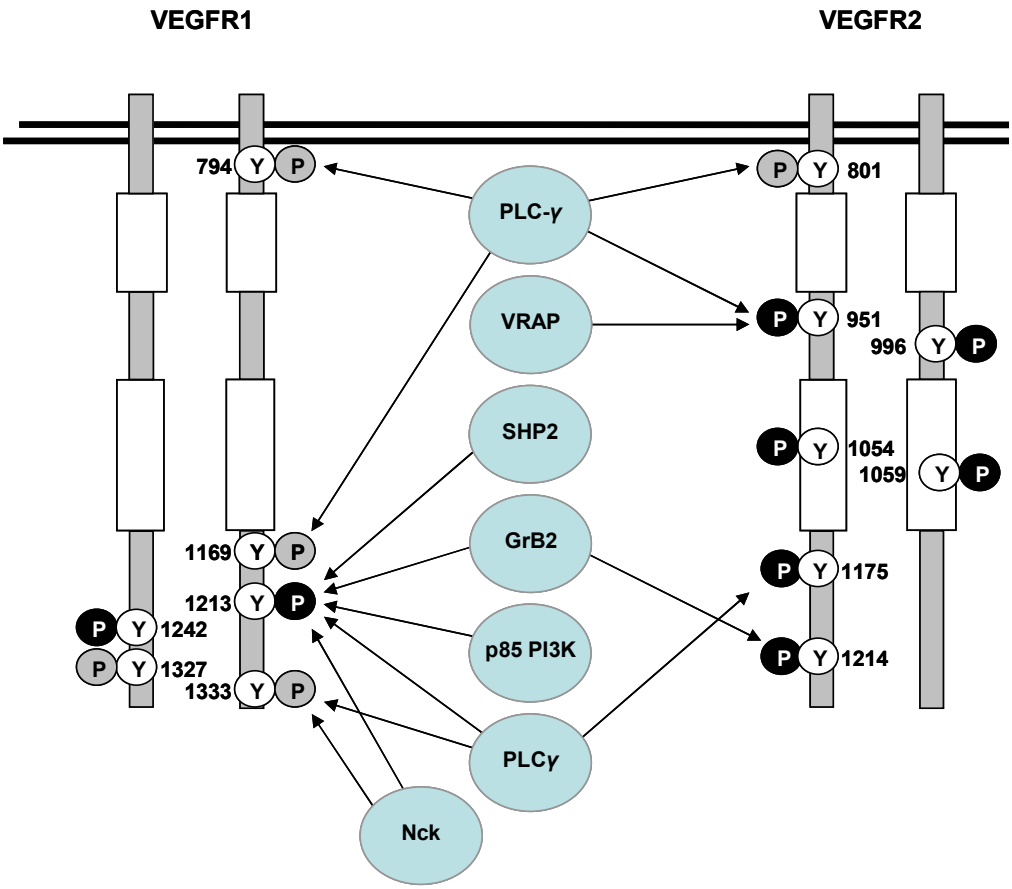


Figure 1.8 Tyrosine phosphorylation sites of VEGFR1 (flt-1) and VEGFR2 (KDR). The intercellular domains of homodimeric complexes of VEGFR1 and VEGFR2 following receptor activation are autophosphorylated at tyrosine residues. Tyrosine residues are indicated by numbers next to an encircled Y, while known autophosphorylation sites (major in black, minor in grey) are indicated by an encircled P. Binding of signal transduction molecules containing an SH2 domain to their respective targets are shown (adapted from Matsumoto *et al.,* 2001).

Conversely Tyr^{951} and Tyr^{996} are located in the kinase insert domain with Tyr^{951} being essential for VEGF-A induced migration (Zeng *et al.,* 2001).

Tyrosine autophosphorylation of VEGFR2 is crucial for recruitment and activation of a variety of signalling complexes required by the cell (Hunter 2000; Schlessinger 2000; see Figure 1.8). Most tyrosine autophosphorylation sites are located in non-catalytic regions of the receptor molecule (Schlessinger 2000) and function as binding sites for molecules with Src homology 2 (SH2) (Kroll *et al.*, 1997; Guo *et al.*, 1995) or phosphotyrosine binding (PTB) domains (Hunter 2000; Schlessinger 2000). These signalling molecules are themselves regulated by phosphorylation and either bind directly to phosphorylated tyrosine domains on the receptor, or to docking/scaffolding proteins (via SH2 binding domains) recruited by the activated receptor (Hunter 2000; Bogatcheva *et al.,* 2003). By this mechanism VEGFR2 activates numerous effector proteins which mediate the various physiological effects of the growth factor VEGF-A (Guo *et al.,* 1995; Schlessinger 2000; Bogatcheva *et al.,* 2000; see Figure 1.9)

Proliferation/migration – activation of MAPK: For EC proliferation, VEGF-A activation of VEGFR2 stimulates PLCγ mediated generation of inositol 1,4,5-triphosphate (IP3) (Wu *et al.,* 2000). This induces protein kinase C (PKC) mobilisation of Ca^{2+} from intracellular stores (Zachary et al., 2001), and activation of the mitogen activated kinase (MAPK) pathway (Kroll *et al.,* 1997). Notably, VEGF-A induces EC proliferation by activating the RAF-MEK-ERK pathway, an unusual feature of which is the requirement for PKC instead of Ras (Takahashi *et al.,* 1999; see Figure 1.9).Survival and NO – PI3K-Akt pathway: Besides proliferation, VEGF-A is a survival factor for EC mediated by VEGFR2-activation of the

phosphatidylinositol-3 kinase - protein kinase B (VEGFR2-PI3K-Akt) pathway (Gerber *et al.,* 1998A; Shiojima *et al.,* 2002; Figure 1.9). PI3K-Akt induces expression the anti-apoptotic proteins Bcl-2 and A1 (Gerber *et al.,* 1998B) which inhibit the activation of upstream caspases pro-apoptotic proteins (Zachary *et al.,* 2001). Furthermore, VEGFR2 activation of PI3K-Akt induces eNOS activity stimulating production of NO in EC (Ku *et al.,*1993; Kroll *et al.,* 1998; Fulton *et al.*, 1999; Dulak *et al.,* 2003) as confirmed by PI3K inhibitors which inhibit VEGF-A induced increase in NO release from endothelial cells (Shiojima *et al.,* 2002). In addition VEGF-A effects on Rho family mediated actin rearrangements associated with EC migration are PI3K-Akt are dependant (Zeng *et al.,* 2001)

Adhesion status and permeability: The signalling activity of VEGFR2 is further influenced by the adhesion status of endothelial cell-cell contacts (Rahimi *et al.,* 1999; Lampugnani *et al.,* 2003). Contact inhibited cells like the vascular endothelium have a reduced responsiveness to VEGF-A induced proliferation and migration when confluent (Rahimi *et al.,* 1999; Lampugnani *et al.,* 1997). This is regulated in part by vascular endothelial cadherin (VE cadherin), a component of the endothelial adherens junction (AJ) (Carmeliet *et al.,* 1999; Venkiteswaran *et al.,* 2002). VEGF-A binding to VEGFR2 phosphorylates components of the AJ resulting in a loss of cadherin mediated endothelial cell-cell adhesion and reduced endothelial barrier function (Cohen *et al.,* 1999; Esser *et al.,* 1998; see Figure 1.10).

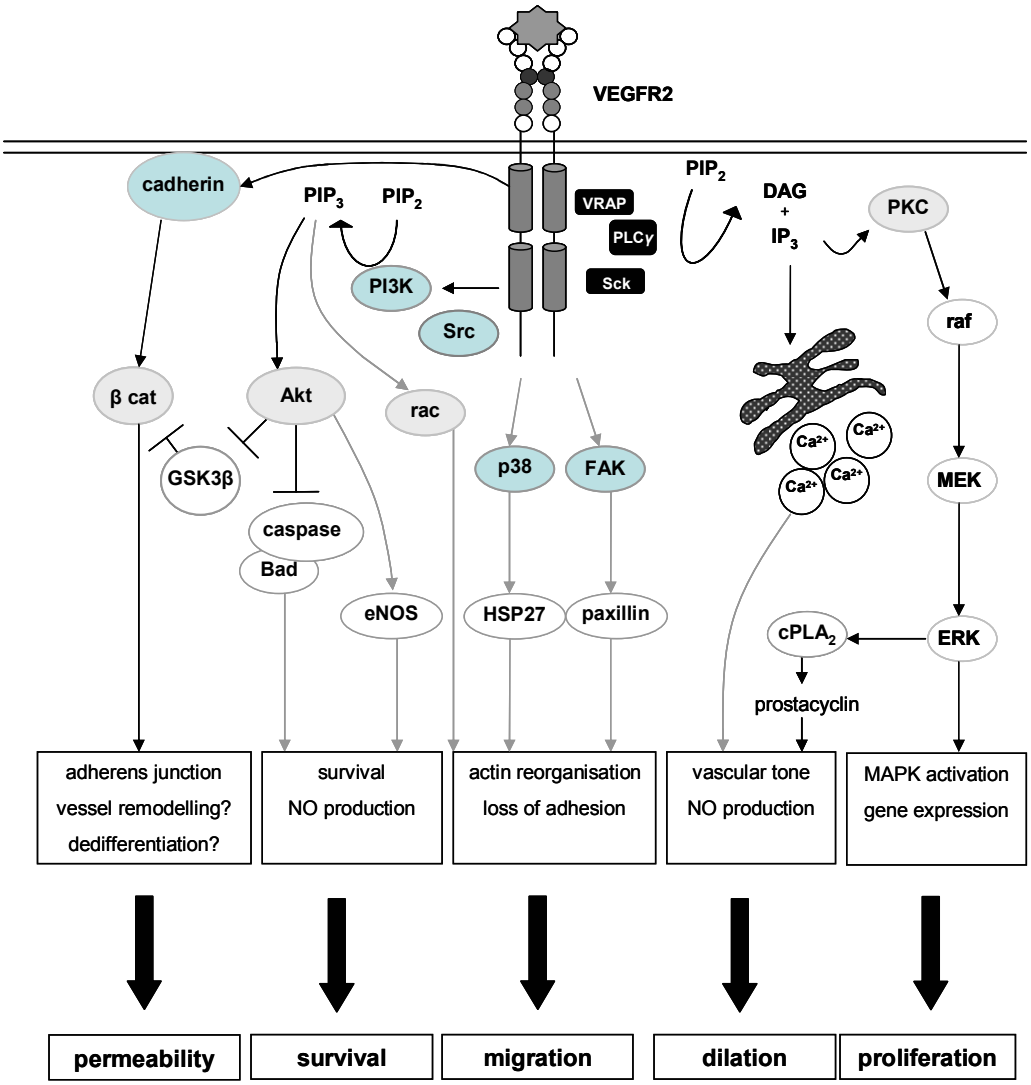


Figure 1.9 Schematic of VEGFR2 (KDR) signal transduction pathways.

VEGF-A binding induces receptor dimerisation and autophosphorylation of tyrosine residues on receptors intracellular domains. Tyrosine phosphorylation activates several SH2 domain containing signalling molecules, either directly following binding (PLCγ) or by indirect mechanism(s) (PI3K, Src) which activate signalling pathways implicated in the regulation of endothelial cell proliferation, migration, vascular homeostasis and permeability (adapted from Matsumoto *et al.,* 2001).

1.2.6 **Role of VEGF-A in the vasculature**

The establishment and maintenance of a vascular supply is a fundamental requirement not only for organ development and differentiation during embryogenesis but also for vascular remodelling, wound healing and reproduction in the adult (Ferrara 1999). The dimeric glycoprotein VEGF-A is a potent, specific mitogen for vascular endothelial cells (Tischer *et al.,* 1989; Millauer *et al.,* 1993) that modulates a range of endothelial cell functions ranging from initial vascular patterning in the developing embryo (Yancopoulos *et al.,* 2000; Jian 2003) to the regulation of angiogenesis and multiple components of vascular homeostasis in the adult (Ku *et al.,* 1993; Bates *et al.,* 1996; Klagsbrun *et al.,* 1996; Ferrara *et al.,* 2003).

1.2.6.1 VEGF-A in the developing embryo

During embryogenesis, mesoderm derived stem cells (hemangioblasts) form blood islands that develop into primitive embryonic endothelial and haematopoietic cells (angioblasts) (Shalaby *et al.,* 1995; Jian 2003). One of the key markers defining the angioblast is the expression of the VEGF-A receptor, VEGFR2 (KDR, Flk-1) (Shalaby *et al.,* 1995; Millauer *et al.,* 1993). The blood island constitutes the basis for the primary vascular plexus which matures through vasculogenesis (the *de novo* formation of blood vessels from angioblasts or stem cells), sprouting angiogenesis (formation of new capillaries from existing vessels) and intussusception (the splitting of the vessel into two daughter vessels) (Shalaby *et al.,* 1995; Millauer *et al.,* 1993; Jian 2003). VEGF-A signalling is an important aspect of this process that not only initiates new vessel formation but also regulates a chain of molecular events that ultimately leads to the development of a mature vascular network (Yancopoulos *et al.,* 2000; Jian 2003). Indeed, VEGF-A is essential to vasculogenesis, with loss of a

single *VEGF-A* allele in mouse models leading to gross developmental abnormalities in the forming vasculature and embryonic death between days 11 and 12 (Table 1.4) (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996). Furthermore, mice lacking either receptor die even earlier between embryonic days 8.5 and 9 (Shalaby *et al.,* 1995; Millauer *et al.,* 1993; Fong *et al.,* 1995). Targeted homozygous inactivation of *VEGF-A* mimics that of VEGFR2 and results in extensive endothelial apoptosis, reduced blood island formation and consequently, major tube vessels fail to develop (Shalaby *et al.,* 1995; Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996; Gale *et al.,* 1999). Conversely, the absence of VEGFR1 results in a preponderance of disorganised endothelial cells that fail to form tubules, although mice lacking only the intracellular tyrosine kinase domain of VEGFR1 survive with a near normal vasculature (Fong *et al.,* 1995).

1.2.6.2 VEGF-A in the adult vasculature

In the adult, VEGF-A plays a central role in the maintenance of vascular homeostasis by regulating multiple functions of blood vessel physiology, including processes involved with angiogenesis (Ferrara *et al.,* 2003), promotion of endothelial cell survival (Gerber *et al.,* 1998A) and regulation of vascular tone (Servos *et al.,* 1999) and permeability (Bates *et al.,* 2002).

The principal mechanism for the formation of new vessels in the adult appears to be angiogenesis (Carmeliet *et al.,* 1996; Yancopoulos *et al.,* 2000). Proteases, produced by activated EC degrade the basement membrane, thus enabling EC migration into the tissue to form a capillary spout (Jian 2003). Here, EC's proliferate and differentiate secreting specific growth factors that attract supporting SMC (pericytes)

gene knockout	stage of vessel development	time of death	cause of lethality
VEGF-A (+/-)	vasculogenesis/ angiogenesis	E11.5	- reduced red blood cell count - defective heart & aorta formation - defective vessel connectivity - defective vessel sprouting
VEGF-A (-/-)	vasculogenesis	E10.5	- absent dorsal aorta - defective EC development
VEGFR1 (*flt-1*)	vasculogenesis	E8.5-9.5	- failure of EC organisation - excessive EC proliferation
VEGFR2 (*flk1*)	vasculogenesis	E8.5-9.5	- failure of EC differentiation - abnormal vessel structures - defective vessel remodelling

Table 1.4 Effects of *VEGF* gene knockout models.

The observed morphological characteristics associated with gene knockouts of *VEGF-A* and its receptors in mouse models of vasculogenesis (from Gale *et al.,* 1999).

which initiate the deposition of a supporting basement membrane required for vessel development (Yancopoulos *et al.,* 2000). VEGF-A has a central role in all these processors processors (Table 1.5) as besides inducing endothelial migration and proliferation (Ferrara *et al.,* 2003) VEGF-A induces EC expression of proteases (interstial collengenase, urokinase-type and tissue–type plasminogen activators) (Pepper *et al.,* 1991), production of cellular energy (stimulates hexose transport) (Pekala *et al.,* 1990), elevated microvascular leakage (Bates *et al.,* 1996) and the continued survival of nascient endothelial cells (Gerber *et al.,* 1998A).

Indeed, a fundamental function of VEGF-A, both in the development of new and maintenance of existing vessel integrity is the promotion of endothelial cell survival through the inhibition of endothelial apoptosis (Gerber *et al.,* 1998A). VEGF-A effects on cell survival have been shown to be mediated by PI3K and Akt (Gerber *et al.,* 1998A; Shiojima *et al.,* 2002; see chapter 1.2.5). The central role of this pathway in controlling endothelial cell viability is illustrated by the activation of PI3K-Akt signalling by several other endothelial cell stimuli associated with the inhibition of apoptosis (Fulton *et al.*, 1999; Shiojima *et al.,* 2002).

In addition, it is well established that VEGF-A acts via VEGFR2 to stimulate the release of endothelial nitric oxide (NO) (Ku *et al.,* 1993; Kroll *et al.,* 1998), both in a Ca^{2+} dependant and Ca^{2+} independent mechanism(s) mediated by Akt activation of endothelial nitric oxide synthase (eNOS) (Gerber *et al.,* 1998A; Fulton *et al.,* 1999). Moreover, VEGF-A produced by vSMC stimulates the production of NO (via eNOS) in the vascular endothelium (Kroll *et al.,* 1998; Dulak *et al.,* 2003). However, NO when released from the endothelium in turn acts on vSMC to produce VEGF-A,

generating a positive feed back 'loop' mechanism central to regulating multiple functions of vessel physiology (Servos *et al.,* 1999, Bundy *et al.,* 2000; Kimura *et al.,* 2003). NO [and prostacyclin (PGI_2)] (Murohara *et al.,* 1998) have been implicated in mediating diverse effects of VEGF-A including the regulation of vascular tone and permeability (Ku *et al.,* 1993; Dulak *et al.,* 2003).

Generation of new blood vessels by angiogenesis is accompanied by increased vascular permeability (Bates *et al.,* 2002), the regulation of which is critical to normal vascular function (Dejana *et al.,* 2001). Indeed elevated expression of VEGF-A associated with pathological angiogenesis (Brown *et al.,* 1993) results in immature vessels with unregulated blood flow (Dvorak *et al.,* 1999). Although the mechanism(s) are unclear, VEGF-A is demonstrated to induce vascular leakage (Dvorak *et al.,* 1999) and endothelial fenestrations in various vascular beds (Roberts *et al.,* 1995), increase hydraulic conductivity in a Ca^{2+} dependant mechanism (Bates *et al.,* 1996) and initiate the rearrangement of the actin cytoskeleton (Shiojima *et al.,* 2002). More recently VEGF-A acting via VEGFR2 was demonstrated to dismantle the endothelial adherens junction (AJ) (Esser *et al.,* 1998) resulting in a loss of cadherin mediated cell-cell adhesion (Kevil *et al.,* 1998) and increased endothelial permeability (Cohen *et al.,* 1999). In addition, dismantling of the AJ is suggested to regulate the responsiveness of EC to VEGF-A signalling, as the loss of cell adhesion is required to deregulate contact inhibition required for endothelial cell proliferation and migration associated with vessel remodelling and angiogenesis (Rahimi *et al.,* 1999; Lampugnani *et al.,* 1997; Dejana *et al* 2000; Dejana *et al.,* 2001).

function	receptor	mediated by	biological effect
proliferation	VEGFR2	MAPK	• DNA synthesis • transcription of *cmyc*, *cfos*, *cyclin D1* • cdk4 activation • translocation of PKC_{α}, PKC_{β}
		PI3K-Akt	• Rho GTPases
		PKC_{α}, PKC_{ζ},PKC_{γ} FAK	• Ca^{2+} mediated increase in cGMP
migration	VEGFR2	MAPK	• phosphorylation of PKC_{γ}, FAK, paxillin
		adherens junction	• phosphorylation of VE-cadherin plackoglobulin, p120, PECAM-1
		PI3K-Akt	• actin filament reorganisation • Rho GTPases
	VEGFR1		• tissue factor, uPA, tPA production
vascular tone	VEGFR2	eNOS activation	• NO production, gene expression • NO mediated ERK activation
		Ca^{2+} mobilisation	• Ca^{2+} mediated increase in cGMP • increase in NO production • increase prostacyclin (PGI_2) production
		PKC	• eNOS phosphorylation
permeability	VEGFR2	raf-1 (ERK)	• PKG mediated increase in permeability • $cPLA_2$ activation
		adherens junction	• phosphorylation of VE-cadherin, β catenin plackoglobulin, p120,
		NO & PGI_2	• elevated permeability
survival	VEGFR2	PI3K- Akt	• cell survival (increased Bcl-2) • inhibition of apopotosis (BAD)

Table 1.5 Function of VEGF-A on adult vascular endothelium.

1.3 VEGF-A, THE ADHERENS JUNCTION AND B-CATENIN

1.3.1 The adherens junction, *Wnt* signalling and β-catenin

The vascular endothelium, a continuous monolayer that lines the lumen of all blood vessels forms an semi-permeable surface that limits passive transfer of cellular and fluid elements between the circulating blood and the vessel wall (Makrides *et al.,* 1998; Dejana *et al.,* 2004; (see chapter 1.4.1). The integrity of the endothelium is maintained by a number of junctional zipper like structures, including tight junctions and the endothelial adherens junction (AJ) (Dejana *et al.,* 2000; Bazzoni *et al.,* 2004), that regulate endothelial barrier function and vessel morphogenesis (Gory-Faure *et al.,* 1999; Carmeliet *et al.,* 1999; Dejana *et al.,* 2001 Bazzoni *et al.,* 2004).

The endothelial AJ is comprised of vascular endothelial cadherin (VE cadherin), a cadherin expressed exclusively on the vascular endothelium (Lampugnani *et al.,* 1992; Breviario *et al.,* 1995), which promotes calcium dependant, extra-cellular, homophilic cell–cell binding (Lampugnani *et al.,* 1992; Dejana 2004). Intra-cellularly, the cadherin cytoplasmic domain interacts with three related proteins belonging to the "armadillo" family of proteins (β-catenin, γ-catenin/plakoglobin and p120) which mediate linkage to the intracellular actin cytoskeleton via α-catenin (Lampugnani *et al.,* 1997; Nelson *et al.,* 2004; Figure 1.10). The structural integrity of the AJ is negatively regulated by phosphorylation (Nelson *et al.,* 2004). Following growth factor activation, tyrosine phosphorylation of β-catenin (Tyr^{654}, Tyr^{142}) by receptor tyrosine kinases or cytoplasmic tyrosine kinases (Wheelock *et al.,* 2003) disrupts the AJ resulting in a loss of cadherin mediated cell-cell adhesion (Dejana 2004) with the release of β-catenin, the transcriptional activator of the Wingless (*Wnt*) signalling pathway into the cytoplasm (Moon *et al.,* 2004; Nelson *et al.,* 2004).

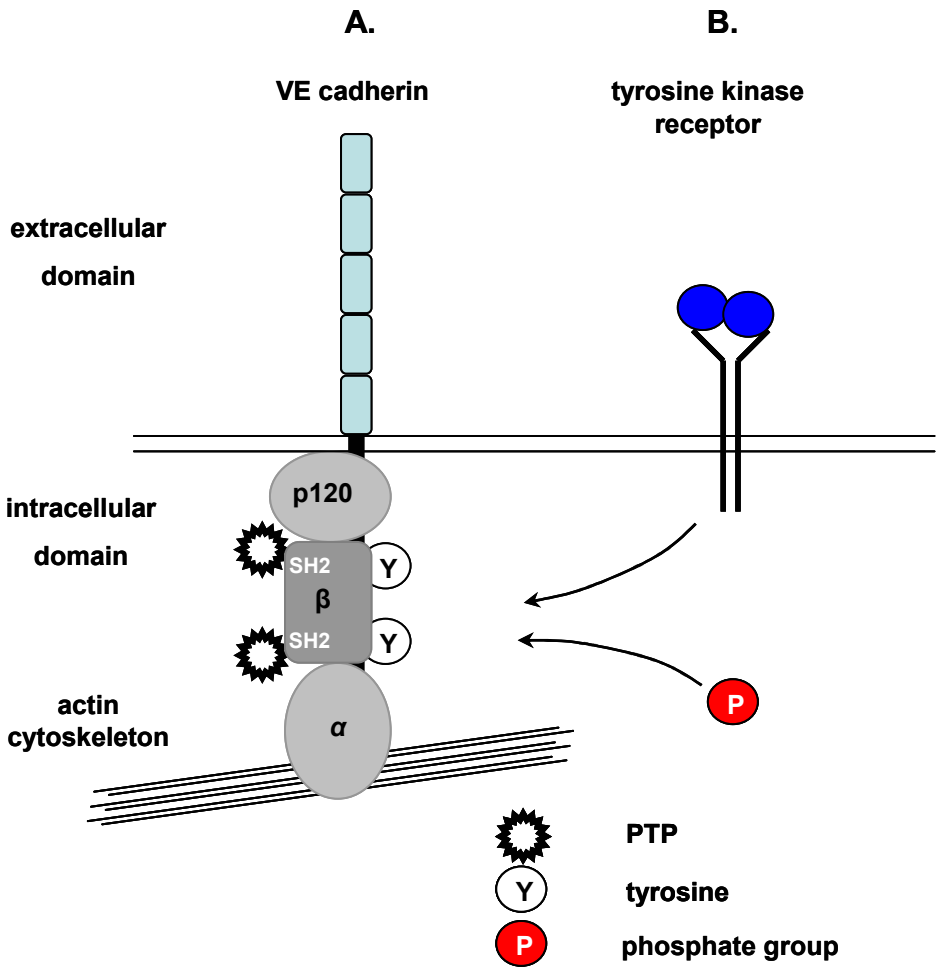


Figure 1.10 Molecular structure of vascular endothelial cadherin.
Schematic illustrating the Ca^{2+} dependent extra cellular domains of VE cadherin (blue rectangles) implicated in mediating cell–cell adhesion in the vascular endothelium. Intracellularly VE cadherin binds the armadillo family of proteins (p120/ γ-catenin, plakoglobin, β-catenin, α-catenin) tethering the AJ to the intracellular cytoskeleton. The AJ is negatively regulated by phosphorylation and the stability of the complex is maintained by tethered protein tyrosine phosphatases (PTP) which bind to conserved protein binding domains (SRC homology 2 domains, SH2) in the intracellular domain (**A**). Activation of tyrosine kinase receptors (like VEGFR2) phosphorylate tyrosine residues in the intracellular domain of VE cadherin, destabilising the complex which leads to the dismantling of the AJ (**B**).

In general, mechanisms that activate tyrosine kinase tend to reduce cadherin mediated adhesion and elevate levels of cytoplasmic β-catenin (Esser *et al.,* 1998; Wright *et al.,* 2002; Wheelock *et al.,* 2003), while activation of protein tyrosine phosphatases (PTP) stabilises the AJ, increasing cadherin mediated adhesion and reducing cytoplasmic β-catenin (Lampugnani *et al.,* 2003; Nelson *et al.,* 2004).

The ability of β-catenin to accumulate in the cytoplasm is tightly regulated by a complex of proteins that control the metabolic stability of β-catenin (Moon *et al.,* 2004). According to the canonical *Wnt* pathway, cytoplasmic β-catenin is targeted for degradation (Aberle *et al.,* 1997) by a complex of serine/threonine kinases [casein kinase I (CKI), glycogen synthase-3β (GSK-3β); (Ikeda *et al.,* 1998; Sakanaka *et al.,* 1999)] in a scaffolding complex of axin (Ikeda *et al.,* 1998; Sakanaka *et al.,* 1998) and the adenomatous polyposis coli protein (APC; Rubinfeld *et al.,* 1996; He *et al.,* 1998; Figure 1.11). However, a large family of secreted glycoproteins (Wnts) stabilise β-catenin by inhibiting GSK-3β (Akiyama 2000; Moon *et al.,* 2004). Binding of a Wnt ligand to Frizzled receptors (Fz), a family of 7 trans-membrane receptors (7-TM), activates Dishevelled (Dsh) in the cytoplasm (Akiyama 2000; Goodwin *et al.,* 2002). Dsh inhibits the GSK-3β containing phosphorylation complex, promoting the accumulation of β-catenin via this, the canonical pathway (Ikeda *et al.,* 1998; Sakanaka *et al.,* 1999; see Figure 1.11).

In addition, a second class of Wnts function via a β-catenin independent mechanism (the *Wnt*/Ca^{2+} pathway), stimulating the release of intracellular calcium and activating calmodulin-dependant protein kinase II (CamKII) and PKC (Kuhl *et al.,* 2000).

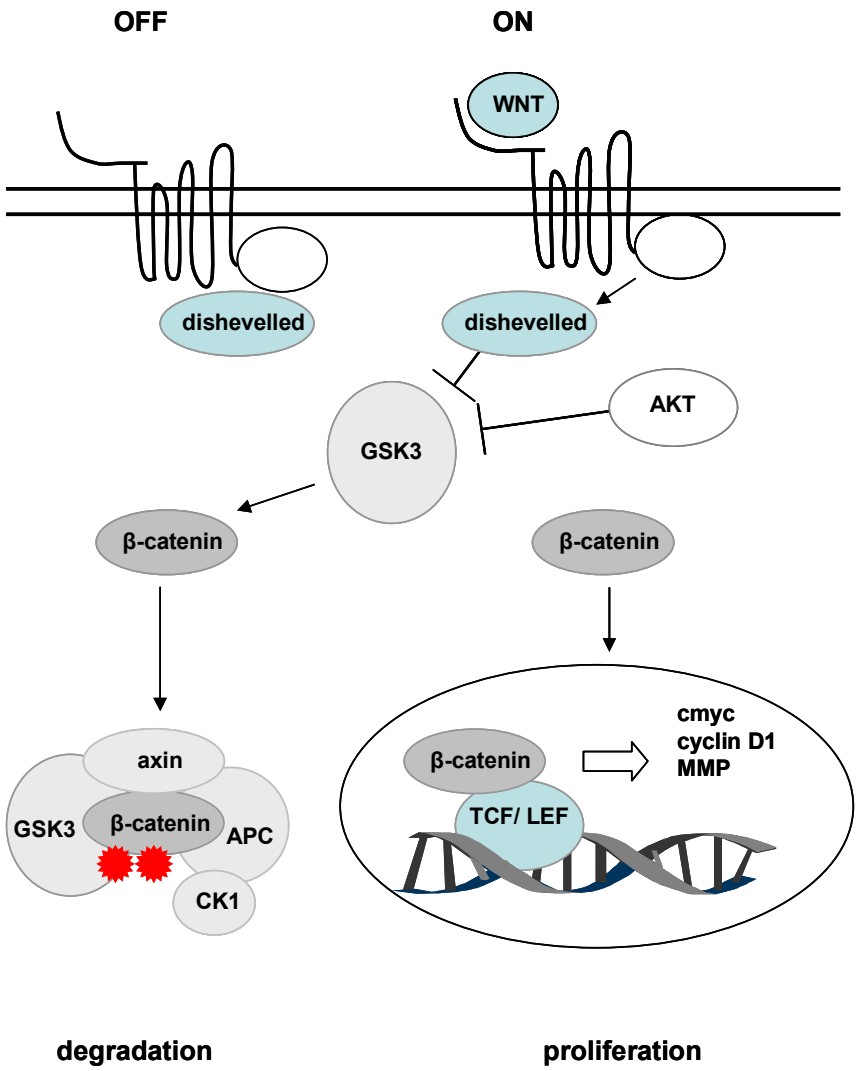


Figure 1.11 β-catenin and the *Wnt* signalling pathway.

In the absence of a Wnt stabalisation signal (**OFF**), free cytoplasmic β-catenin is rapidly phosphorylated by GSK-3β, and marked for degradation via ubiquitin mediated proteolysis. Conversely, the presence of a Wnt signal acting through the Frizzled receptor (**ON**) inactivates GSK-3β which allows the accumulation of β-catenin in the cytoplasm. Free β-catenin translocates to the nucleus which results in TCF/LEF mediated gene transcription. Interestingly, Akt, the mediator of VEGF-A induced EC survival induced via VEGFR2 similarly inhibits GSK-3β which may result in the accumulation of β-catenin in the cell.

When stabilised, cytoplasmic β-catenin translocates to the nucleus (Huber *et al.* 1996; Blankesteijn *et al.,* 2001), binds the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription complexes (Behrens *et al.,* 1996; He *et al.,* 1998; Shtutman *et al.,* 1999) and initiates the transcription of numerous genes involved in cellular proliferation and differentiation potentially implicated with vascular remodelling including *c-myc* (*MYC)*, *cyclin D1* (*CND1*) and the matrix metalloproteases *MMP2*, and *MMP7* (Gumbiner 1995; Behrens *et al.,* 1996; He *et al.,* 1998; Shtutman *et al.,* 1999). In addition to regulating endothelial barrier function therefore, the AJ is suggested to regulate the activity of β-catenin signalling by maintaining bound β-catenin in the AJ and thus preventing its translocation to the nucleus for gene transcription (Gottardi *et al.,* 2004; Dejana 2004). The precise role of β-catenin in the vasculature is unclear however, although there is an absolute requirement for the protein as the conditional inactivation of the β-catenin gene causes a defective vascular pattern with fragile vessels (Cattelino *et al.*, 2003).

1.3.2 **VEGF-A, VEGFR2 and the adherens junction**

The mechanism(s) through which VEGF-A mediates vascular permeability are not clearly understood, although like most other mediators of permeability including NO, thrombin and histamine (Andriopoulou *et al.,* 1999; Bates *et al.,* 2002; Dejana *et al.,* 2001), VEGF-A increases paracellular permeability of the vascular endothelium by disrupting the endothelial AJ (Kevil *et al.,* 1998). VEGF-A binding to VEGFR2 tyrosine phosphorylates components of the AJ (Esser *et al.,* 1998; Cohen *et al.,* 1999) resulting in a loss of cadherin mediated endothelial cell-cell adhesion and reduced endothelial barrier function (Cohen *et al.,* 1999). Dismantling of the AJ therefore results in increased endothelial paracellular permeability (Kevil *et al.,* 1998, Dejana

et al., 1997) and the release of β-catenin into the cytoplasm (Cohen *et al.,* 1999; Figure 1.12). Furthermore, dismantling of the AJ regulates the responsiveness of EC to VEGF-A signalling, as the loss of cell adhesion is required to deregulate contact inhibition required for endothelial cell proliferation, migration and permeability associated with vessel remodelling and angiogenesis (Rahimi *et al.,* 1999; Lampugnani *et al.,* 1997; Dejana *et al* 2000; Dejana *et al.,* 2001).

Following VEGF-A activation, VEGFR2 associates with VE cadherin (Rahimi *et al.,* 1999; Wright *et al.,* 2002; Zanetti *et al.,* 2002) forming a complex that localises to the intracellular junction (Carmeliet *et al.,* 1999; Rahimi *et al.,* 1999; Lampugnani *et al.,* 2003). The formation of VE cadherin VEGFR2 complex is dependant on the β-catenin binding domain of VE cadherin (amino acids 703-784; Carmeliet *et al.,* 1999) as demonstrated by the failure of truncated VE cadherin to form the complex which inhibits phosphorylation of VEGFR2 and VEGF-A mediated survival via PI3K (Carmeliet *et al.,* 1999; Lampugnani *et al.,* 2003).

As protein tyrosine phosphatases (PTP) are tethered at intracellular junctions (Young *et al.,* 2003; Nawroth *et al.,* 2002), VE cadherin is suggested to act as a platform facilitating interaction between VEGFR2 and PTP found in the AJ (Zanetti *et al.,* 2002; Lampugnani *et al.,* 2003). This close proximity to PTP in the AJ is demonstrated to modulate the activity of VEGFR2 signalling (Rahimi *et al.,* 1999; Zanetti *et al.,* 2002) by directing VEGFR2 signalling to specific pathways (Lampugnani *et al.,* 2003). The pathway selected is determined by recruitment of adapter proteins (see chapter 1.2.5) to phosphorylated tyrosine residues on the intracellular VEGFR2 domain (Carmeliet *et al.,* 1999; Lampugnani *et al.,* 2003).

Consequently, VEGF-A activation induces VEGFR2 mediated EC survival via Akt (Carmeliet *et al.,* 1999; Gerber *et al.,* 1998A), while simultaneously inhibiting PLCγ mediated proliferation (Lampugnani *et al.,* 2003) in confluent EC. Furthermore, the association of PTP with the VE cadherin VEGFR2 complex can dephosphorylate active tyrosine motifs on intracellular domains of VEGFR2, essentially deactivating the receptor (Zanetti *et al.,* 2002; Lampugnani *et al.,* 2003 see Figure 1.12).

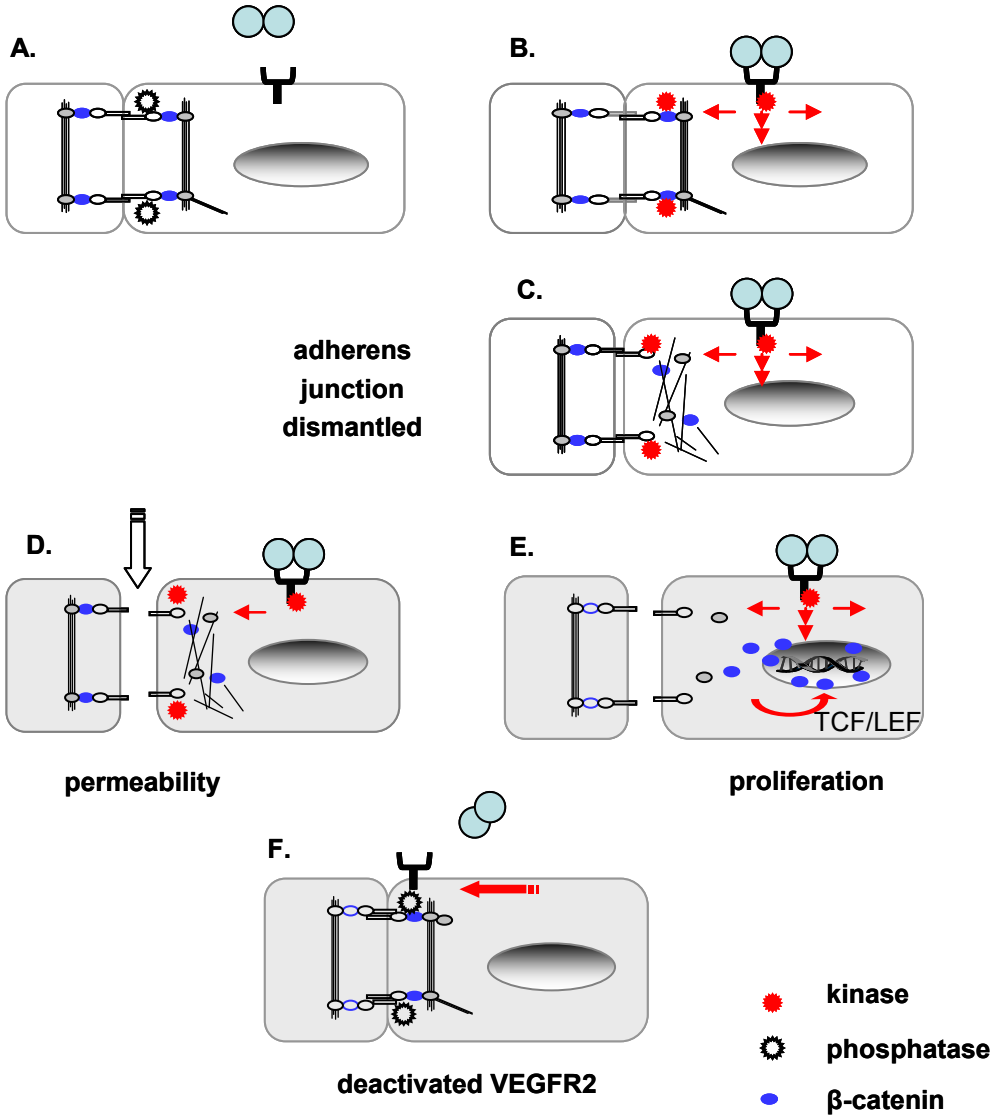


Figure 1.12 Dismantling of endothelial AJ by VEGF-A signalling.

Confluent endothelial cells present a semi-permeable surface to the vessel lumen (**A**). VEGFR2 mediated signalling tyrosine phosphorylates components of the AJ (red dots) (**B**) leading to the dismantling of the AJ (**C**). Dismantling of the AJ increases endothelial paracellular permeability (**D**) and releases β-catenin from the cadherin /catenin complex (blue dots). β-catenin, if not degraded translocates to the nucleus and activates numerous genes involved with proliferation and vessel wall remodelling via the TCF/LEF transcription complex (**E**). Deactivation of VEGFR2 and dephosporylation of the catenin/cadherin components by protein tyrosine phosphatases (PTP) co-localised here is thought to initiate the restabilisation of the at the cadherin / catenin complex complex which reforms the AJ (**F**).

1.4 VEGF AND PRIMARY VARICOSE VEINS

The underlying clinical abnormality associated with primary VVs is venous incompetence, principally in the greater saphenous system (Labropoulos *et al.,* 1999; Ruckley *et al.,* 2002) which results in venous hypertension, perturbed blood flow (turbulence, stagnation and reflux), dilatation and abnormal vein wall remodelling (Browse *et al.,* 1999; Labropoulos *et al.,* 1999; Nicolaides 2000; Badier-Commander *et al.,* 2000; Ruckley *et al.,* 2002). Historically the initiation of varicosity is thought to occur following a "mechanical" problem such as descending valvular incompetence (Golledge *et al.,* 2003; see chapter 1.1.4). Clear evidence, however refuting Trendelenburg's model of 'descending valvular incompetence' as the primary cause of varicosity, is published extensively in the literature (Abu-owen *et al.,* 1994; Labropoulos *et al.,* 2000; Hollingsworth *et al.,* 2001B).

The introduction of colour flow duplex ultrasound examination in the diagnosis of venous incompetence, has further challenged this assumption and contributed to our understanding of the presentation of disease in relation to functional characteristics of the varicosity like the underlying venous incompetence (Labropoulos *et al.,* 1997; Allen *et al.,* 2000; Ruckley *et al.,* 2002; Cooper *et al.,* 2003). Observations from these studies suggest that the development of varicosity may occur by a process of progressive "spreading incompetence" potentially originating and spreading from a local focal point(s) (Jones *et al.,* 1999; Labropoulos *et al.,* 1999; Cooper *et al.,* 2003). This is substantiated by observations that varicosity is often patchy and irregular, with varicose segments of vessel wall interspersed with apparently normal segments (Badier-Commader *et al.,* 2001; Wali *et al.,* 2001B).

At the cellular level, histological analysis of the morphology and composition of cellular and matrix components of the vein wall confirms the heterogeneity nature of the varicose vessel wall when compared to that of a normal vein (see chapter 1.1.6). These distinct patterns of morphological and structural alterations are considered to be the result of aberrant vessel-wall remodelling (Gandhi *et al.*, 1993; Venturi *et al.,* 1996; Badier-Commader *et al.,* 2000). Furthermore, it has been demonstrated that the processors involved are influenced by the same growth factors implicated in the regulation of normal vascular homeostasis (Yancopoulos *et al.,* 2000; Badier-Commader *et al.,* 2001). This suggests that an aberrant molecular control of, or perhaps an inappropriate response to mediators of normal vascular homeostasis like VEGF-A may potentially pre-dispose to the development of primary VVs.

VEGF-A, acting in concert with nitric oxide (NO; Servos *et al.,* 1999) plays a central role in the maintenance of vascular homeostasis by regulating multiple functions of blood vessel physiology including vascular dilatation and permeability (Ku *et al.,* 1993; Bates *et al.,* 1996; Klagsbrun *et al.,* 1996; Ferrara *et al.,* 2003). Previous studies demonstrated that patients with primary VVs had a loss of release of VEGF-A following a mild experimentally induced stasis (Hollingsworth *et al.,* 2001A). Furthermore plasma NO was reduced significantly suggesting perhaps a fault in VEGF maintenance of vascular reactivity (Hollingsworth *et al.,* 2001A). The loss of release of VEGF-A in response to a stimuli like disturbed blood flow or elevated intra luminal pressure, may suggest a fault in the VEGF system in VVs. Although VVs have the ability, albeit a reduced ability, to respond (contract or dilate) to exogenously added mediators of vascular tone *in vitro* (Schuller-Petrovic *et al.,* 1997; Brunner *et al.,* 2001), fewer investigations into the ability of the vein wall itself to

produce mediators of vascular homeostasis like VEGF-A, have as of yet, been undertaken. Moreover, as the control of molecules like VEGF-A, involved in maintaining vascular reactivity is fundamental in the regulation of homeostasis, examination of the activity of such molecules in association to the pattern of disease presentation, may help to elucidate the mechanism(s) underlying varicogenesis.

1.5 **HYPOTHESIS**

Valve reflux is considered a principal cause of primary VVs, although there is no consensus as to whether valve incompetence is the primary initiating event in the pathogenesis of varicose disease or may merely occur secondary to vein wall dilation (Beebe-Dimer *et al.,* 2005). The proposal here, is that potentially the initiation and development of primary VVs may arise following a loss of regulation/control of VEGF-A locally by cells in the vessel wall, a molecule intimately implicated in maintaining vascular reactivity.

Functional characteristics like disturbed blood flow within the 'pre-varicose' vein may lead to a temporary hypertensive intra-luminal state (Pearson *et al.,* 1998) due either to an anatomical abnormality or simply reduced return blood flow due to a lack of physical movement (Michiels *et al.*, 1997). The appropriate homeostatic response for increased pressure however, should be to relieve 'symptoms' by mediating dilatation (and/or an increase in vessel wall permeability) to prevent venous reflux/stasis (Luscher 1991; Browse *et al.,* 1999; Recek 2004) and as such the release of dilatory mediators like VEGF-A and NO would be expected (Michiels *et al.*, 1997; Servos *et al.,* 1999; Hollingsworth *et al.,* 2001A). If not resolved, a localised

abundance of VEGF-A combined with a continuous chronic stimulation to dilate may result in an altered sensitivity by components of the vein wall to available VEGF-A.

Overtime local homeostatic mechanism(s) may become unregulated (Sho *et al.*, 2003) which together with other factors associated with CVI, like hypoxia may drive further production of VEGF-A (Pearson *et al.,* 1998; Michiels *et al.,* 1996). The vein, potentially having lost the ability to mediate an appropriate molecular response/control to VEGF-A may start to respond aberrantly, ultimately resulting in the vessel wall remodelling and histological changes seen in VVs.

1.5.1 **Aims:**

To investigate this hypothesis, the first requirement is to demonstrate that varicose vessels have the capability to produce both VEGF-A and its receptors. As described in chapter 1.2.2 VEGF-A is regulated on multiple levels, but is transcriptionally upregulated in vascular SMC in response to a number of factors including elevated venous pressure, disturbed blood flow and hypoxia (Pearson *et al.,* 1998, Shay-Salit *et al.,* 2002, Sho *et al.*, 2003).

Aim 1: To investigate the pattern of transcription of *VEGF*-A and its receptors in relation to the underlying venous incompetence: Here, the patterns of gene transcription of *VEGF-A* and its receptors *VEGFR2, VEGFR1, s.flt-1* were examined in VVs in relation to the underlying venous incompetence (from duplex scan), to investigate potential associations between a functional characteristic of varicose disease like disturbed blood flow (Browse *et al.*, 1999; Ruckley *et al.,* 2002) with alterations in gene activity.

If the vein can produce VEGF-A and its receptors, then dysfunction of venous reactivity and vascular remodelling observed in VVs may be due to an inability of the vessel wall to respond appropriately to available VEGF-A. As described in chapter 1.2.5 VEGF-A mediates vascular reactivity and homeostasis via a number of intracellular signalling pathways. If as suggested, the action of VEGF-A is disturbed with the development of primary VVs, then alterations to the proper functioning of VEGF-A should affect its normal role in homeostasis *i.e.* the regulation of dilatation and permeability.

Aim 2. To examine the pattern of activity of β-catenin gene transcription and protein expression in relation to the underlying venous incompetence in VVs and control vein samples. Although the mechanisms by which VEGF-A induces vascular permeability are not clearly understood, VEGF-A binding results in the dismantling of the AJ resulting in a loss of endothelial cell adhesion, with increased paracellular permeability and the release of β-catenin to the cytoplasm (see chapters 1.2.6 and 1.3.2). If VEGF-A signalling is disturbed with the onset of varicose disease, which does indeed occur by a process of progressive "spreading incompetence" (Jones *et al.,* 1999; Cooper *et al.,* 2003), then alterations to the pattern of expression of β-catenin may similarly associate to the patterns of disease presentation observed. Notably, β-catenin if not degraded may translocate to the nucleus and activate numerous genes involved in proliferation and cellular differentiation associated with vascular remodelling (see chapter 1.3.1). So in addition, *c-myc* and *cyclin D1* gene transcription was similarly examined to investigate possible associations between β-catenin and the transcriptional activation of its target genes in relation to the patterns of disease presentation.

Aim 3: To examine baseline levels of plasma s.flt-1 in the GSV and its release following a mild experimentally induced venous hypertension and whether release is affected by varicosity. Finally in controlling vascular reactivity the endothelium must be able to switch off cellular response(s) to VEGF-A. A lack of attenuation of VEGF-A signalling by mechanisms modulating either VEGF-A availability, or interaction with the VEGF receptors may potentially facilitate continued VEGF-A signal activation. The soluble form of VEGFR1, s.flt-1 is proposed to modulate the activity of VEGFR2 (Kendall *et al.,* 1996A; Graubert *et al.,* 2001) and so attenuates the action of VEGF-A signalling (Kendall *et al.,* 1993; Belgore *et al.*, 2000). Here, the release of s.flt in response to elevated venous pressure was examined to determine if a mechanism implicated in the modulation of VEGF-A action was intact in individuals with VVs

Chapter 2

MATERIALS AND METHODS

2.1 SAMPLE COLLECTION AND PROCESSING

2.1.1 Ethics approval and patient selection

Approval was obtained from the Joint UCL/UCLH Committee on Ethics of Human Research prior to commencement of the study for the collection and use of all human tissue used in this investigation (see Appendix 1). The diagnosis of primary VVs was made by the referring surgeon, and confirmed by the vascular technologist using venous Duplex ultrasound scans as clinically evident VVs, C.E.A.P. Grade II or above (Porter *et al.,* 1995). Patients who had either previous surgery for VVs, evidence of current or previous DVT or elected not to consent to the use of blood or tissue were excluded from the study.

2.1.2 Sample collection

Greater Saphenous vein: Samples of VVs were collected at operation for sapheno-femoral ligation and stripping of GSV as treatment for primary VVs. Examination of reflux at the sapheno-femoral junction (SFJ) and GSV was investigated with patients standing and reported as incompetent only when the duration of reflux exceeded 0.5s (Porter *et al.,* 1995). The position of incompetent venous segments was cross checked against the duplex scan report following removal of the vein. Prior to operation, a record was made from the duplex scan of patterns of venous incompetence and distribution of varicosities. Samples of control vein were obtained from patients undergoing cardiac bypass procedures and who had no symptoms or clinically evident signs of varicose disease in either limb; veins were confirmed to be normal by a pre-operative duplex scan, showing that the vessel was disease-free and without valvular incompetence in any segment.

Following removal, the GSV sample was immediately placed in cold (4°C) Soltran kidney perfusate solution (Baxter Ltd UK, appendix 3) and transported to the laboratory for processing. Varicose GSV sample was dissected into five anatomical segments starting from the sapheno-femoral junction, as proximal (P), proximal to mid (P-M), mid (M), mid to distal (M-D) and distal (D) (Figure 2.1). The final number of segments harvested from each vein was dependant on the length of the vein that was stripped, with only those stripped to below knee providing five segments (average length of vein section was between 5 to 10 cm, depending in part on the extent of tortuosity present within the segment). With this procedure the competency of the segment (reflux as determined by colour flow Doppler) could be correlated to the specific anatomical position of the segment along the length of the vein. The author thanks Mr David Cooper for the analysis and alignments of vessel segment competencies to that determined by duplex scan and for the collection of vein samples.

Between 100 to 300mg approximately 5 cmsontrol or varicose GSV tissue was transferred to 2 ml cryovials (Nalgene, UK) and flash frozen in liquid nitrogen (LN_2, BOC Ltd, UK). The vein segments were stored under vapour phase of liquid nitrogen until subsequent processing for RNA and protein.

Venous stasis and collection of blood plasma: All individuals enrolled in this study were non-smokers receiving no medication. Individuals (control subjects or patients with primary VVs) were rested supine for 15 minutes. A foot vein was cannulated using a G23 butterfly and once positioned, was flushed through with 5ml of saline for injection. Individuals were then rested supine for a further 10 minutes before 5ml of

peripheral blood was collected (into EDTA; Vacutainer®) from the foot vein (followed by a further 5ml flush of saline for injection). A control blood sample was removed from the arm immediately afterwards. Whilst remaining supine, a sphygmomanometer cuff was placed directly below the knee and inflated to 90-95mmHg to prompt a mild induced venous stasis (Hollingsworth *et al.,* 2001A). Ten minutes following application of the cuff, a further 5ml of peripheral blood was obtained from the foot vein as before. The author thanks Mr Tang for the collection of bloods as described.

Samples of peripheral blood were then separated through Ficoll® (Sigma-Aldrich Ltd, UK) at 400g for 25 minutes at room temperature. The plasma was transferred to 2ml cryovials (Nalgene, UK) and stored at -20°C until further analysis.

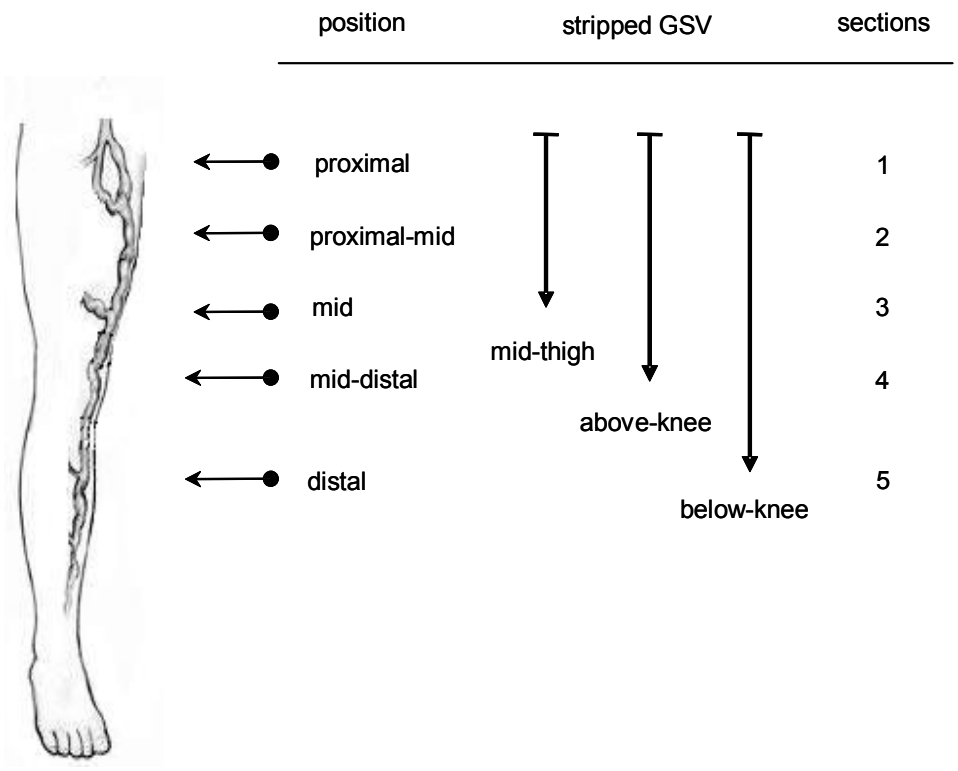


Figure 2.1 Position of vein segments obtained for analysis.

Position from the SFJ (position); section number (section) and extent of stripping of the GSV undertaken at operation, with corresponding numbering of sections and respective segmental position from the SFJ (stripped GSV). Section numbering and position were performed to match anatomical positions of sections taken for analysis, and to enable cross-reference to duplex scans to determine competency (reflux or not) of the section at the position taken.

2.2 EXTRACTION OF RNA AND PROTEIN FROM VEINS

2.2.1 Background

Although numerous techniques exist for the isolation of RNA and protein from tissue, they all essentially share the following common attributes: i) membrane disruption and cell lysis, ii) inhibition of endogenous ribonuclease (RNAse) and protease activity, iii) the separation of cellular constituents and contaminants, iv) recovery of intact, uncontaminated RNA or protein. The first two steps tend to present the most problems as all tissues contain endogenous RNAse and proteases sequestered within cellular compartments. Disruption of cell membranes during cell lysis releases RNAse and proteases, which if not inactivated immediately can cause the rapid degradation of cellular RNA and protein (Chomczynski 1993).

Most current methods of RNA isolation therefore are based on disrupting the tissue in the presence of strong denaturing or chaotropic chemicals. High molarity guanidinium solutions of thiocyanate or chloride salts are among the most effective protein denaturants and consequently inhibitors of RNAse activity available and have found application in most current protocols for RNA purification procedures (Chomczynski *et al.,* 1987). Furthermore, tissue homogenates in guanidinium thiocyanate can be manipulated at room temperature and the hazard of inadvertently introducing exogenous RNAse during processing is of little importance (Ambion product insert). Although techniques for the isolation of protein are normally tailored to the application for which the protein is be utilised, the method described below allows for the isolation of RNA, DNA and protein from the same sample (Chomczynski 1993). The protein obtained is suitable for semi-quantitative analysis by western blot.

Here, total RNA was extracted from crushed vein tissue segments using TRI Reagent™ (Sigma-Aldrich Ltd, UK) according to the method previously described by Chomczynski. TRI Reagent™, a mixture of guanidine thiocyanate and phenol in a mono-phasic solution, effectively dissolves RNA, DNA and protein on homogenisation of tissue (Chomczynski 1993). After addition of chloroform followed by differential centrifugation, the mixture separates into three phases: the top aqueous phase containing RNA, the interphase containing DNA and an organic phase containing cellular protein. Each component can then be purified following the initial separation of the phases.

2.2.2 **Protocol: Extraction of RNA using TRI Reagent™**

A ceramic mortar was cooled to less than -80°C with liquid nitrogen. A frozen vein segment of known mass was quickly removed from liquid nitrogen storage and placed immediately in the mortar filled with liquid nitrogen. The tissue was crushed to a fine powder, using a pre-chilled (-80°C) pestle, topping up the mixture with liquid nitrogen as required to ensure the tissue/powder did not thaw. After complete homogenisation, 1 ml of TRI Reagent™ (Sigma-Aldrich Ltd, UK) was added per 50-100 mg of tissue. The tissue powder/TRI Reagent™ mixture was frozen with liquid nitrogen, transferred to 50 ml Bluemax tubes (Sterilin, UK) and defrosted on the bench. The defrosted tissue/ TRI Reagent™ homogenate was mixed thoroughly and incubated for 10 minutes at room temperature to facilitate complete dissociation of nucleoprotein complexes. Insoluble cell debris was collected by centrifugation at 2000g for 10 minutes at room temperature. 1 ml of the homogenate supernatant was transferred to 1.5 ml sterile eppendorf tubes (1 ml per tube) containing 0.2 ml chloroform (BDH Biosciences, UK). The sample was mixed thoroughly for 15

seconds and incubated on the bench for 10 minutes at room temperature. The phases were separated by centrifugation at 12 000g for 15 minutes at 4°C. The aqueous phase containing total RNA was transferred to a sterile 1.5 ml eppendorf tube without disturbing the interphase (containing DNA) to minimise possible DNA contamination of the RNA. The interphase and organic phase were stored at -20°C for subsequent isolation of cellular protein. Total RNA was precipitated from solution with the addition of 0.5 ml isopropanol (BDH biosciences, UK) and collected by centrifugation at 12000 g for 10 minutes at 4°C. The supernatant was discarded and the pellet washed with 1 ml of 75% v/v ethanol by centrifugation at 7500 g for 5 minutes at 4°C. The ethanol wash was discarded and the RNA pellet was air-dried for 3 minutes at room temperature, taking care to prevent complete desiccation of the RNA pellet. The pellet was suspended in 30-50 µl in DNAse and RNAse free water (Qiagen Ltd. UK) by repeated pipetting with a micropipette and stored at -20°C for 24 hours. The RNA solution was defrosted and the concentration of isolated RNA was determined by spectrophotometric analysis according to the Beer Lambert Law relating concentration of a solution to its absorbance.

$$\textbf{Concentration (µg/µl)} = \mathbf{A_{260}} \times \varepsilon \times \mathbf{Df}$$

where A_{260} = absorbance at 280nm
ε = extinction coefficient for RNA ($M^{-1}\,cm^{-1}$)
Df = dilution factor (1:50)

The RNA was reverse transcribed to complimentary DNA or stored at -20°C.

2.2.3 **Protocol: Extraction of total protein**

DNA contained in the interphase was precipitated with the addition of 0.3 ml 100% ethanol and collected by centrifugation at 2000 g for 5 minutes at 4°C. The supernatant (containing total protein) was transferred to a clean 15 ml Bluemax (Marathon Labs Ltd, UK) and stored on ice. Total protein was precipitated from the phenol-ethanol supernatant with 1.5 ml cold 100 % isopropanol. The sample was mixed thoroughly by inversion and incubated on ice for a minimum of 20 minutes. Precipitated proteins were collected by centrifugation at 12000 g for 10 minutes at 4°C. The supernatant was discarded and the protein pellet washed with 1.5 ml 0.3M guanidine-hydrochloride/95% ethanol solution. During each wash the protein pellet was incubated for a minimum of 20 minutes at room temperature, then collected by centrifugation at 7500 g for 5 minutes at 4°C. After three washes the protein pellet was washed with 2 ml 100% ethanol, vortexed and incubated at room temperature for 20 minutes. The protein pellet was collected by centrifugation at 7500 g for 5 minutes at 4°C, the ethanol wash was discarded and the protein pellet dried on the bench. The dried pellet was crushed with a metal rod and total protein was extracted with 100 µl 1% sodium dodecyl sulphate per 100 mg tissue (SDS, Sigma-Aldrich Ltd, UK). The extract was incubated at 50°C for 30 minutes followed by centrifugation at 10 000 g for 10minutes at 4°C to remove insoluble debris. The cleared supernatant containing total protein was transferred to a clean 1.5 ml sterile eppendorf tube and stored on ice. Protein concentration was determined by the method of Bradford and protein aliquots stored at -20°C (Bradford 1976).

2.3 ANALYSIS OF GENE TRANSCRIPTION

2.3.1 Reverse Transcription

Reverse transcription (RT), the process of copying RNA to complimentary DNA (cDNA) is essentially an *in vitro* adaptation of the mechanism by which RNA viruses replicate following transduction *in vivo* (Lewin 1990). In the RT reaction, RNA is used as template to reverse transcribe a complimentary strand of DNA by the action of a RNA directed DNA polymerase. The RNA is initially denatured by heat and then cooled rapidly in the presence of a nucleotide primer. As the temperature cools the primer binds to its target complimentary sequence on the RNA template. The addition of a mixture of deoxyribonucleotides (dNTP) and a DNA polymerase enzyme in an appropriate buffer initiates the polymerisation reaction resulting in the production of a complimentary DNA strand.

Enzymes used in RT: The two RNA-dependant DNA polymerase enzymes routinely used for RT are derived from the avian myoblastosis virus (AMV) or the moloney murine leukaemia virus (MMLV) (Biosource Primer). Here we have utilised MMLV-RT, a DNA polymerase that reverse transcribes single stranded RNA following priming with an oligonucleotide primer. MMLV-RT can tolerate temperatures up to a maximum of 42°C and like all DNA polymerases, has an absolute requirement for magnesium ions (Mg^{2+}) to function (reaction buffer contains 15mM $MgCl_2$). The recombinant enzyme is isolated from *E.coli* expressing a portion of the *pol* gene of MMLV-RT on a plasmid (Sigma-Aldrich product sheet).

Primers used in RT: Nucleotide primers confer the specificity of the technique by binding to their complimentary target sequence on the RNA template. Bound primer

serves as a binding site for RT polymerases which then initiate strand synthesis by incorporating nucleotides into the growing chain in a 5' to 3' direction. RT primers generally fall into one of three categories depending on the application.

Oligo dT primers: Primers comprising repeated thymine nucleotides being complimentary to the polyadenylation signal on the 3' end of mammalian message RNA (mRNA) are used to reverse transcribe mRNA. As oligo(dT)'s bind specifically to mRNA species only, reverse transcription using these primers selectively enriches for genes that have been transcribed and has wide application in analysis of gene expression. Although Oligo(dT)'s often produce a full length cDNA product, transcribing the message from the 'tail end' can sometimes be problematic, for example if the mRNA sequence is either very long or have significant secondary structure

Random primers: Random primers, short random repeats of six to ten nucleotides, bind complimentary sequences on all RNA species and therefore reverse transcribe total cellular RNA (mRNA as well as ribosomal and transfer RNA species). As random primers bind to the template at multiple sites they are not as constrained by long mRNA sequences (or secondary structure) and therefore produce a higher proportion of the 5' region of the RNA sequence (Bioscience Primer). However random primers only produce cDNA's of partial length.

Gene specific primers: Finally gene specific primers can be used to reverse transcribe a segment of the mRNA for a specific gene only. Gene specific primers tend to be

used for the detection of low copy number genes but have the disadvantage in that only that one gene sequence can be investigated with the cDNA produced.

2.3.2 **Protocol: Reverse Transcription**

Reverse transcription was performed as described in the product sheet for MMLV-RT supplied with slight modifications (Table 2.1). Total RNA (4 µg) was aliquoted in a 0.5 ml Eppendorf tube in the presence of 2 µl random primers (125 ng/µl) and made up to a total volume of 24µl with deionised RNAse free water. The tube was place in a Progene thermal cycler (Techne Ltd, UK) and the sample was heat denatured at 70°C for 10 minutes. The sample was removed and cooled on ice for 4 minutes to allow the primers to anneal to the RNA template. A mastermix containing 8 µl 5x first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 4 µl of the reducing agent DTT (100 mM) and 2 µl dNTP mix (10 mM of each deoxyribonucleotide: adenine, thymine, cytosine and guanine) was added, the solution was mixed and incubated at 42°C for 2 minutes. 2 µl of recombinant MMLV-RT, 200 u/µl, Sigma-Aldrich Ltd) was added and cDNA was synthesised during the following 50 minute incubated at 42°C. The heat sensitive MMLV-RT was deactivated with heat at 75°C for 15 minutes, and the newly synthesised cDNA was stored at -20°C.

Reagent	Concentration / Quantity	Volume (µl)	Total
Total RNA	4 µg	X	
Random primers	125 ng/µl	2	
dd H_2O		Y	24 µl
- Heat to 70°C for 10 minutes			
- Chill on ice for 5 minutes, then add 14 µl of mastermix			
Mastermix			
First strand buffer	5x	8	
DTT	100 mM	4	
dNTP mix	10 mM each of dATP, dTTP, dGTP, dCTP	2	14 µl
- Incubate at 42°C for 2 minutes, then add			
MMLV-RT	200 u/µl	2	
- incubate at 42°C for 50 minutes			
- denature MMLV-RT with heat at 75°C for 10 minutes			
Total volume			**40 µl**

Table 2.1 Protocol for reverse transcription reaction.
(5x first strand buffer, see Appendix 4)

2.3.3 **Polymerase Chain Reaction**

The polymerase chain reaction (PCR) is an invaluable technique for the analysis of gene expression and has revolutionised the field of molecular biology since it was first described (Mullis *et al.*, 1986). PCR, like reverse transcription is an *in vitro* method for the enzymatic synthesis of DNA. However, unlike reverse transcription which produces only one faithful copy of RNA, PCR amplifies the target DNA sequence exponentially, producing millions of copies of the DNA sequence of interest (Bosher 2000). PCR is essentially a cycling reaction which utilises a combination of alternating primer hybridisation and DNA replication reactions to amplify a sequence of DNA (Figure 2.2). Each cycle is composed of three individual steps, a denaturation step, an annealing step and the strand elongation step. In the denaturing step, the cDNA template is denatured (with heat) to separate the DNA double helix into its two component strands, thereby exposing the purine and pyrimidine base structure contained in the genetic code. The reaction is then cooled during the annealing step to a temperature which promotes optimal binding of primers to their complimentary DNA sequence. The bound primers serve as an initiation site for the binding of a heat stable DNA polymerase. In the strand elongation step, the temperature is raise to the optimum for the DNA polymerase which synthesizes a new DNA strand in a 5'-3' direction using the existing strand as the template. The DNA duplex consisting of the original template and newly synthesised DNA strand are then denatured again and a new cycle of amplification is initiated. Exponential amplification of the DNA sequence of interest results, as with each successive cycle both the original template as well as the newly synthesized strand can be primed for new strand synthesis.

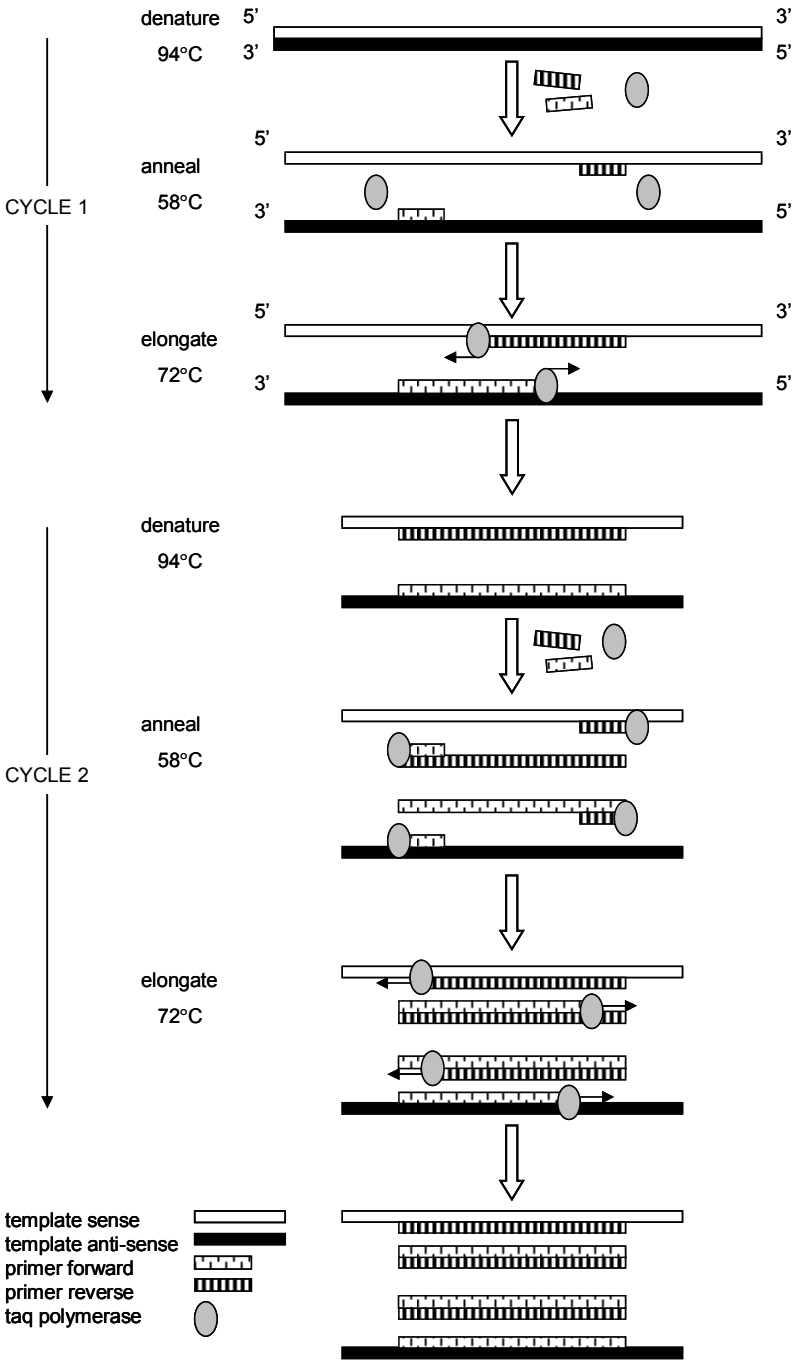


Figure 2.2 Schematic of the polymerase chain reaction (PCR).

In cycle 1, a double stranded DNA template is denatured in the presence of complementary primers to facilitate binding at specific binding sites. The annealed primer serves as an attachment site for a DNA dependant polymerase which synthesises a new DNA strand. As the newly synthesised strands from cycle 1 can now act as template in cycle 2, cycle 2 starts with four templates. This selective amplification of the target sequence yields millions of copies, all of the same size as determined by the target sequence determined by the primer pair.

In a standard PCR reaction, each cycle is repeated thirty to forty times which is sufficient to increase the number of copies of the sequence being examined a million fold (Bosher 2000). Amplification by PCR is particularly useful therefore when a limiting amount of RNA is available for analysis. Conversely, in situations when a quantitative analysis is to be undertaken, variation in the amounts of starting RNA (cDNA) can have a substantial effect on the amount of final product produced (Kendall *et al.,* 1996B). Furthermore, the kinetics of the PCR reaction has a narrow linear range, being in dependent in part on the amount of template used at the start of the reaction (Higuchi *et al.,* 1993). These problems need to be addressed when using PCR to determine the level of gene transcription in a cell *i.e.* to control for differing amounts of RNA (cDNA) in the samples and the kinetics of the PCR reaction itself.

Two strategies are routinely used to make an analysis of the level of gene transcription. The first makes use of a known quantity of an engineered DNA sequence that contains the same primer binding sites as the sequence of interest but yields a different size product (Cross 1995). Both DNA standard and experimental sample are amplified in the same reaction tube and the amount of experimental product produced is determined from the amount of internal standard used initially to give a quantitative result (Heid *et al.,* 1996). Alternatively, a "house keeping" gene, a gene constituently expressed in the cell, is used as an "internal standard" (Vandesompele *et al.,* 2002). Here the amount of experimental product is determined as a ratio to the amount of "house keeping" gene product produced, to yield a semi-quantitative result of the level of gene transcription in the sample (Cross 1995). The use of primers to a control transcript, in our case glyceraldehyde-3-phosphate dehydrogenase (GAPDH), shows that each sample investigated has undergone

equivalent reverse transcription to cDNA and that the cDNA is amplifiable during PCR. Since the control sequence is not used to define absolute levels of mRNA, differences in primer efficiencies between the control and test primers are not important (Dallman *et al.,* 2005). Furthermore, this approach can be readily applied to the analysis of transcription of multiple genes from the same sample using the same "internal standard".

A typical PCR reaction requires the following components: target DNA, oligonucleotide primers, deoxynucleotide triphosphates (dNTP), a thermostable DNA polymerase, and magnesium in a suitable reaction buffer. As the stringency of the reaction is affected by many of the above parameters the reagent concentrations required for optimal amplification were determined empirically for each set of primers, in combination with the initial amount of starting template used. Important points to note in the optimisation of PCR are discussed.

Primer design: Successful PCR depends more on the design of appropriate primers than any other component in the reaction. The specificity and sensitivity of PCR is achieved by exploiting the stringent bonding characteristics of purine-pyrimidine base pairing in DNA by using sequence specific primer pairs. The PCR primer therefore not only dictates the specific DNA sequence to be amplified, but also defines other characteristics of the reaction including size of product and annealing temperature used. Poorly designed primers can both increase the amplification of spurious gene products by non-specific binding and decrease the product yield by encouraging the formation of primer dimers. A few parameters to consider when designing primers are discussed below:

Primer Length: Primers of between 18 to 30 nucleotides in length provide good specificity to detect an unique target sequence, while minimising the probability of primer dimmer formation (Dallman *et al*., 2005). Both primers of the pair should ideally contain similar numbers of each nucleotide, while the GC content should be between 40-60% (Dallman *et al*., 2005).

Primer sequence: The sequence of the primer should be complimentary to a unique region of the gene being investigated. To minimize the probability of non-specific binding, the primer should not target DNA sequences that contain polypurine (A, G) or polypyrimidine (T, C) sequence strings, GC rich regions or repetitive DNA sequences (*Alu* elements). For optimal amplification the binding of the 3' end of the primer is critical as polymerisation occurs in a 5'-3' direction. There should be no mismatch between the primer and target sequence within the last 3-6 nucleotides of the 3' end. Further, stipulating the terminal 3' nucleotide is a guanine or cytosine (GC clamp) improves the efficiency of primer binding due to the stability of the 3' bond (3 hydrogen bonds). Finally there should be minimal self-complimentary within each primer (3' intra-homology) as well as between the two primer pairs (inter-homology) to reduce the formation of artefacts and primer dimers.

Primer melting temperature: The melting temperature (Tm) of the primer is defined as the temperature at which 50% of a primer in solution is hybridised to the target sequence (Bosher, 2000). The specificity of the hybrid formation between primer and target is influenced by both the geometry of the bases responsible for hydrogen bonding (adenine and thymine, 2 bonds: guanine and cytosine, 3 bonds), as well as the electrostatic interaction of the phosphate groups which tend to destabilise the double stranded molecule (Lewin 1990). The Tm of a PCR primer is dependant on the number of GC bases in the primer sequence and the salt concentration of the

buffer solution. Consequently, the Tm is a good estimate of the annealing temperature used for PCR, as it reflects the temperature required to break half the hydrogen bonds between template and primer. Of the numerous methods used to determine the Tm in the PCR the most accurate is a thermodynamic calculation based on entropy, enthalpy, free energy and temperature, "the nearest neighbour" formula (Bosher 2000).

$$\mathbf{Tm = H[S + R \ln (C/4)] - 273.15 + 16.6 \log 10[K+]}$$

where H = enthalpy
S = entropy for helix formation
R = molar gas constant
C = concentration of primer

For simplicity, the Tm for nucleotides of up to 18 nucleotides in length can be estimated with the following formula as described by Wallace.

$$\mathbf{Tm = [(number\ of\ G + C) \times 4] + [(number\ of\ A + T) \times 2] - 4}$$

Template sequence: As mentioned, each primer of the pair should recognise a segment of the template sequence that is unique. To reduce the amplification of genomic DNA, the primer pair is designed to either anneal to target sequence located on an intron exon boundary in the mRNA, or in two different exons (separated by an intron). Consequently, amplification of contaminating genomic DNA will give a different sized product to the target mRNA. In practice, with the development of new algorithms most primers are routinely chosen with primer picking software.

All primers used in this study, unless stated otherwise, were selected with Primer 3, a software package from the Whitehead Institute, available free of charge on the internet (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3). The homology of

the selected primers were analysed against all published sequences in the human genome using the Basic Alignment Sequence Homology Tool (BLAST) available from NCBI on the internet (http://www.ncbi.nih.gov). Table 2.2 lists the molecules investigated, their chromosomal location and the NCBI reference sequences used to select the primers as detailed in this study. A complete list of the primer sequences, including parameters like the targeted exon/intron, Tm and product size are listed in Table 2.3 while additional characteristics are presented for *VEGF-A* (Figure 2.3), *sflt-1* (Figure 2.4) and all genes (Appendix 3).

Annealing temperature: The specificity of binding of the primer to the template target sequence is determined by the annealing temperature of the PCR reaction. The annealing temperature, as discussed above is determined by the Tm of the primers. As the annealing temperature approaches the Tm, the stringency of the reaction is increased. This reduces the possibility of primers binding to non-specific sequence on the template DNA. The annealing temperature is generally kept 2-4°C below the lowest Tm of the primer pair used in the reaction. The length of time allowed for annealing is also important and is usually between 30-60 seconds. Since DNA polymerase do have some activity at lower temperatures, a long annealing time increases the probability of amplification of non-specific products. In this study, the annealing time was set to 30 seconds as standard.

Elongation: The efficiency of Taq polymerase is approximately 1000bp per minute at 72°C. The time allowed during elongation to synthesize the new DNA strand is calculated from the expected size of the product, as defined by the primer pair. As all the primers used in the study were designed to produce products of less than 500bp the extension time for all primer sets was set to 45 s at 72°C (750bp).

Gene	symbol	chromosome	ref sequence	size (base pair)
GAP- 3	*GAPDH*	12p13	NM_002046.2	1315
VEGF-A	*VEGF*	6p12	NM_003376	784
β-catenin	*CTNNB1*	3p21	NM_001904.1	3357
c-myc	*MYC*	8q24	NM_002467.2	2168
cyclin D1	*CCND1*	11q13	NM_053056	4288
VEGFR2	*KDR*	4q11-4q12	NM_002253	5832
VEGFR1	*FLT-1*	13q12	NM_002019.1	7090
s.flt-1	*sFLT-1*	13q12	U 01134	2061

Table 2.2 Characteristics of the genes examined in this study.
(see Appendix 3).

Primer name	exon	sequence (5' - 3')	bp	Tm °C	product bp
GAP406 for	6	CTCATGACCACAGTCCATGC	20	**60**	406
GAP406 rev	8	TGACAAAGTGGTCGTTGATT	20	**60**	
GAP452 for	8	GACCACAGTCCATGCCATCACT	22	**68**	452
GAP452 rev	9	TCCACCACCCTGTTGCTGTAG	21	**67**	
VEGF for	3	CATCCTGTGTGCCCCTGATG	20	**62**	*v121*-243
VEGF rev	8	TTCCTCCTGCCCGGCTCAC	19	**64**	*v165*-375
βCAT7 for	7	CAAGCAGAGTGCTGAAGGTG	20	**64**	250
βCAT7 rev	9	ATTCCAGCTGCACAGGTGAC	20	**65**	
βCAT11 for	11	GTTGTACCGGAGCCCTTCAC	20	**65**	243
βCAT11 rev	13	ATGTCGCCACACCTTCATTC	20	**65**	
cmyc for	3	CCTACCCTCTAACGACAGC	19	**59**	248
cmyc rev	4	CTCTGACCTTTTGCCAGGAG	20	**64**	
cyclin D1 for	2	TCTAAGATGAAGGAGACCATC	21	**57**	354
cyclin D1 rev	4	GCGGTAGTAGGACAGGAAGTTGTT	24	**65**	
VEGFR2 for	26	AGACTTTGAGCATGGAAG	18	**55**	312
VEGFR2 rev	29	CCATTCCACCAAAAGATG	18	**59**	
VEGFR1 for	13	TCATGAATGTTTCCCTGCAA	20	**60**	277
VEGFR1 rev	15	GTGCTGCTTCCTGGTCCTAA	20	**60**	
s.flt-1 rev	int 13	TTTGTTGCAGTGCTCACCTC	20	**60**	119

Table 2.3 Primer pairs used in this study.

Primers were designed with Primer 3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3) from reference sequence or taken from the literature [*GAP452* and *c-myc* (Zhihua *et al.,* 2003), *cyclin D1* (Gumbiner *et al.,* 1999) and *VEGF* (Tokunaga *et al.,* 1998)].

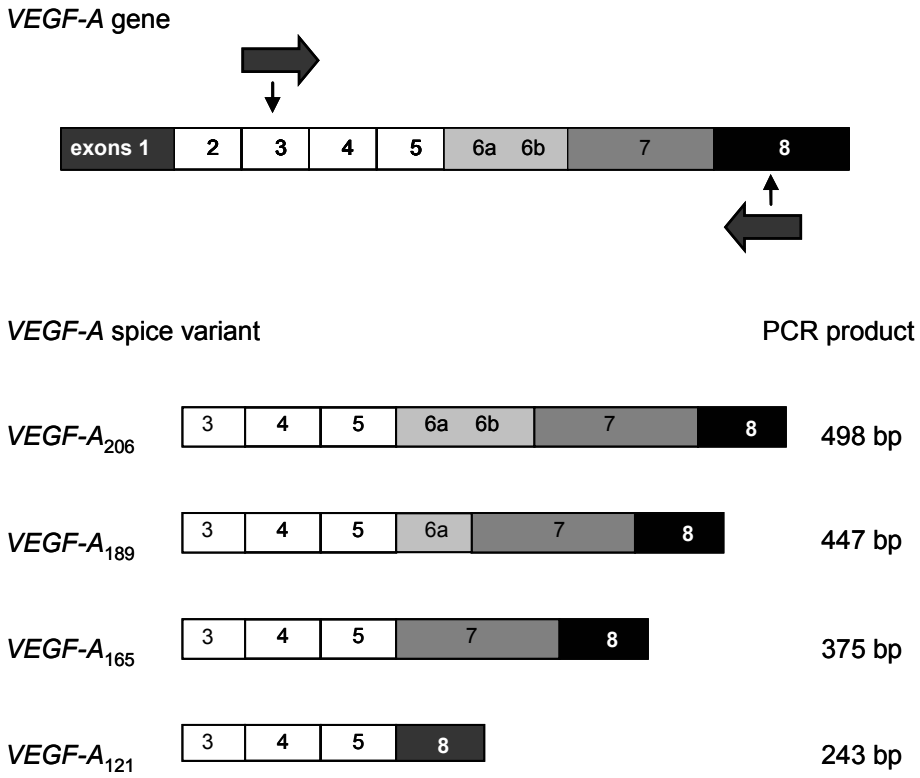


Figure 2.3 Primer pair for VEGF detects all possible splice variants of *VEGF-A*. The 13.4 kB *VEGF-A* gene is coded by eight exons that produce a mature mRNA of 784 bp. Although four different isoforms of *VEGF-A* exist in the cell, all produced by alternative splicing of the mature mRNA, most cells preferentially express *VEGF-A*$_{121\text{-}189}$. *VEGF-A*$_{189}$ lacks 51 bp in exon 6, while total loss of exon 6 produces *VEGF-A*$_{165}$ mRNA and loss of both exon 6 and 7 produces *VEGF-A*$_{121}$. The primers designed for *VEGF-A* were based on those originally used by Tokunaga *et al.,* 1998 and detect all *VEGF-A* transcripts produced by the cell.

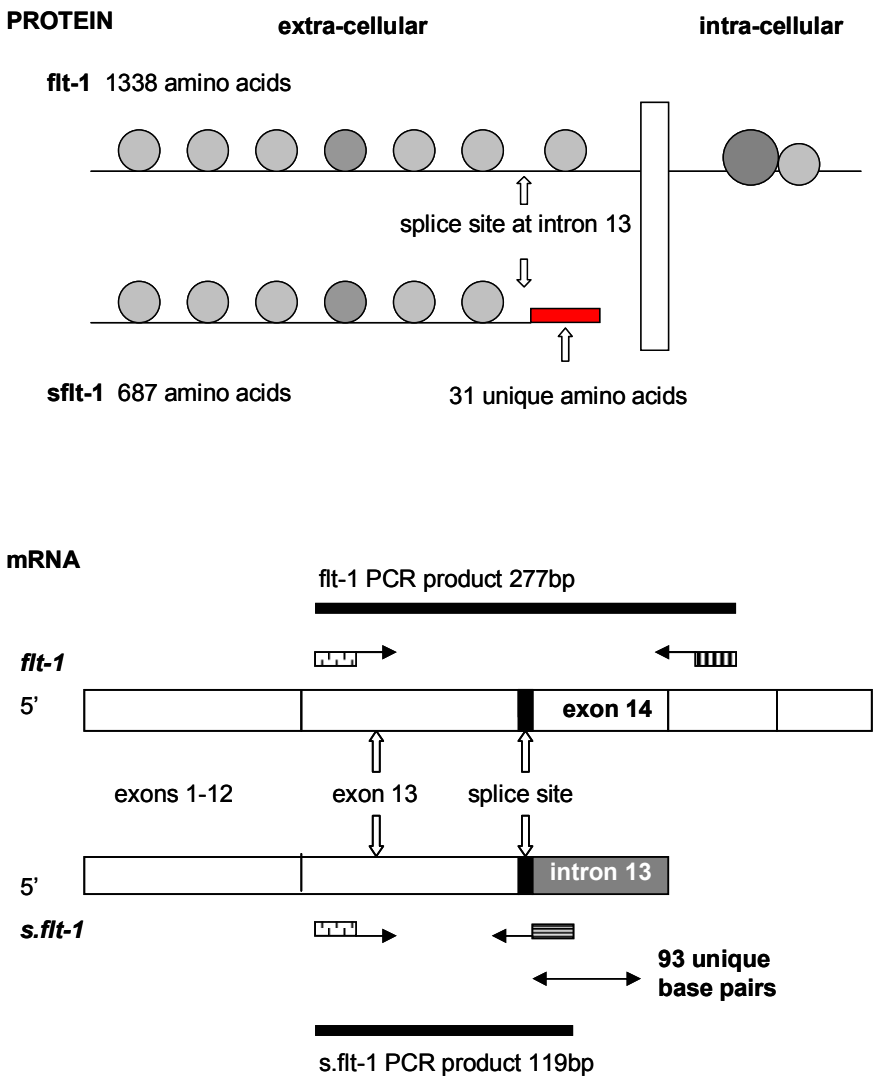


Figure 2.4 Amplification of *s.flt-1* requires a unique intronic primer.

The large 194.7 kb *VEGFR1* gene is coded by 30 exons that produce a mature mRNA of 7.090 kb. Alternative splicing of *VEGFR1* results in two mRNA transcripts, one gives rise to the membrane bound receptor, while a premature termination at a polyadenylation site of intron 13 results in the soluble isoform (*s.flt-1*) (Kendall *et al.,* 1993; Kondo *et al.,* 1998). Consequently, 93 base pairs of *s.flt-1* are unique and can be used to differentiate the mRNA of the two isoforms.

Magnesium chloride: DNA polymerases have an absolute requirement of magnesium ions (Mg^{2+}) to function properly. The concentration of Mg^{2+} therefore can be utilised to alter the stringency of the PCR reaction by modulating the activity of the polymerase. The Mg^{2+} concentration affects the efficiency (rate of synthesis) and fidelity (error rate) of the polymerisation reaction. Low Mg^{2+} concentration increases the stringency of the PCR reaction. Reactions at high stringency decrease both non-specific binding of primer to the template, as well as the fidelity of polymerisation. Conversely, an excess of Mg^{2+} decreases the stringency of the reaction. Reactions at low stringency contribute to the production of artefacts (increased non-specific binding of the primer) and decrease the fidelity (increased error rate) of the polymerase. Taq polymerase, the most widely utilised heat resistant DNA polymerase for PCR, demonstrates optimal activity at 1.2-1.3 mM free Mg^{2+}. The concentration of free Mg^{2+} in PCR however, is affected by the concentration of both deoxyribonucleotides (dNTP) and primer in the reaction. In solution there is equimolar binding of free Mg^{2+} to dNTP and changes in either the primer or dNTP concentration will therefore affect the concentration of available Mg^{2+}. All PCR in this study used 2 mM $MgCl_2$, 200 μM dNTP mix (200 μM of each dNTP) and 1 μM of each primer (see Table 2.4). The theoretical concentration of free Mg^{2+} therefore was approximately 1.2 mM, approaching the optimal Mg^{2+} concentration required for Taq polymerase.

DNA polymerase: There are a large number of different DNA dependant polymerases for PCR, all of which however characteristically synthesise DNA in a 5'-3' direction from the free 3' hydroxyl group. For PCR, polymerases can generally be grouped according to their "proof-reading" capability, as defined by the presence

or absence of a 3'-5' exonuclease. Polymerases, like Taq polymerase that do not contain 3'-5' exonuclease activity have high efficiency (rate of synthesis) but low fidelity (high error rate) and incorporate an incorrect nucleotide with a frequency of 1 in 10^4 bases. Conversely, polymerases that do contain 3'-5' exonuclease activity (Pfu, Pwo) make few errors during polymerisation (high fidelity) and are used in applications like cloning where the integrity of the product sequence is important. As mentioned polymerases have activity at temperatures below their optimum. The consequences of this enzyme property requires special consideration when setting up the reaction, as DNA template in the absence of dNTP can be degraded by "proofreading" polymerases. Furthermore Taq polymerase has 5'-3' exonuclease activity, which in the presence of excess enzyme may increase the production of artefact by degrading the DNA template. To circumvent this problem use is made of a "hotstart technique". Here the components of the reaction are added together in the absence of Taq polymerase. The PCR reaction is started and the polymerase is only added to the reaction prior to the initial elongation step.

As analysis of gene transcription used here was semi-quantitative, the fidelity of the enzyme was not of primary importance. REDTaq™ polymerase, a Taq polymerase mixed with an inert red tracer dye was obtained from Sigma-Aldrich at a concentration of 1u/µl. Here, 0.5 units REDTaq™ polymerase per reaction was used for PCR, with one unit defined as the quantity of enzyme required to incorporate 10nmol of total dNTP into acid precipitable DNA for 30 minutes at 74°C. The enzyme was added as the last component according to the "hotstart" method.

2.3.4 **Protocol: Polymerase Chain Reaction**

PCR was performed essentially as described in the product insert supplied with REDTaq™ Polymerase from Sigma with slight modifications, in a total reaction volume of 50 µl (Table 2.4). To minimise pipetting errors all reagents were prepared as a mastermix (1). With the exception of REDTaq™ reagents were all vortexed to disperse any salt gradients formed on freezing, before the matermix was prepared. On completion, the mastermix was vortexed then aliquoted to individual, autoclaved 0.5ml Eppendorf tubes, prior to the addition of cDNA template (2). A positive and negative control was included for each primer set. The positive control (cDNA from GSV isolation) had previously been demonstrated to produce a PCR product with the reaction conditions used. The negative control simply substituted template cDNA with ddH_2O to control for possible contamination. The reagents were mixed by centrifugation at 2000 g for 1 minute and placed in a Techne thermocycler. The reaction was initiated with a four minute denaturation cycle at 94°C, followed by the addition of REDTaq™ mix (3) using a "hotstart" at 65°C. The PCR was amplified for 35 cycles (Table 2.5). After 35 cycles the products were incubated at 72°C for 10 minutes and chilled to 4°C. The amount of gene product produced following PCR amplification was determined by agarose gel electrophoresis.

mastermix	component	volume (µl)	concentration
1	ddH_2O	32.5 or 33.5	-
	10 mM dNTP mix	1.0	200 µM
	25 mM $MgCl_2$	4.0	2 mM
	10x reaction buffer	4.3	1x
	100 pmol Forward Primer	0.5	1 µM
	100 pmol Reverse Primer	0.5	1 µM
volume		**43 or 44**	
2	**cDNA (100 ng/µl)**	**2 or 1**	0.1-0.2 µg/µl
3	ddH_2O	4	-
	10x reaction buffer	0.5	1x
	REDTaq 1u/µl	0.5	0.01u/µl
total	**volume**	**50**	

Table 2.4 The protocol for PCR reaction.

PCR step	temperature	time (seconds)
denature	94°C	30
anneal	56-60°C	30
elongate	72°C	45

Table 2.5 PCR cycling parameters.

2.3.5 **Electrophoresis**

Electrophoresis involves the movement of a charge molecule in an applied electric field. Migration of the molecule occurs in a liquid medium (a buffer salt solution of known pH and ionic strength) following the application of an external voltage (V). The degree of movement of the charged molecule in the applied electric field is termed its electrophoretic mobility (μ). μ is a function of the size, net charge and structural confirmation of the molecule and is described by the following relationship:

$$\mu = \frac{Q}{6\pi\eta r} \times V$$

where Q = net charge on the molecule
r = radius of the molecule
η = viscosity of the liquid medium
V = applied voltage

In a medium of constant viscosity and at a constant applied voltage, the movement of a charged molecule is governed by its charge-to-size ratio (Q/r), as the charge to size ratio increases (the molecule size decreases) the migration of the molecule increases. In practice though, resolution of molecules in solution is poor and use is made of an inert solid support matrices (including starch, agarose and polyacrylamide) to improve separation. A support matrix is a three dimensional polymer network which forms pores of uniform size, with the size of the pore being determined by the concentration of the gel polymer used. The porosity of the gel however, presents a different effective viscosity of the medium to each molecule through a "molecular sieving" effect (as determined by the size of the individual molecule itself). Due to molecular sieving effect of the gel matrix therefore, gel electrophoresis discriminates between molecules by better reflecting differences in molecular size. For molecules

of a similar shape electrophoretic mobility through a gel matrix is inversely proportional to the log of the molecular weight (Morris *et al.,* 1971).

DNA agarose gel electrophoresis: The most common inert supports used in DNA electrophoresis are agarose or polyacrylamide. Agarose, (a linear polysaccharide extract) is used for separation of large molecular weight fragments. Acrylamide gels are used for smaller fragments and can resolve differences in DNA to a single base pair. The optimal size of DNA fragment that can be resolved using agarose is determined by the porosity of the gel. The advantage of an agarose matrix is that the porosity of the gel can be controlled simply by changing the percentage of agarose used in the mixture. DNA carries a net negative charge due to the negatively charged phosphate groups in the sugar-phosphate backbone. Because of the phosphate groups, the charge of a DNA fragment is almost directly proportional to its molecular weight during electrophoresis. Resolution of DNA fragments therefore occurs through the "molecular sieving" effect of the gel as defined by pore size. With agarose electrophoresis, DNA fragments are resolved according to size.

The migration of DNA through agarose gels is not linear but inversely proportional to the log of the molecular weight of the fragment. For example the separation between a 100bp and a 1000bp fragment is greater than that between 1000bp and 2000bp fragment. The migration of DNA through agarose is dependant not only on size, but is also affected by the conformation of the molecule. In general, the more compact the structure the quicker it will migrate, as compact structures can negotiate the pores in the agarose better than a linear molecule of equal size. However, the electrophoretic mobility of DNA can be used to approximate the size of a DNA

fragment, if the migration of the fragment is compared to that of control DNA fragments of known size.

2.3.6 **Protocol: Agarose gel electrophoresis**

Agarose gels were made with RNAse free agarose (Merk, UK) using a Tris Borate EDTA buffer (TBE, pH 8). A 2% gel was made by dissolving 3 mg of agarose in 150 ml of 0.5x TBE (5x TBE stock: 54 g Tris base, 27.5 g Boric acid, 20 ml 0.5M EDTA per litre) in an Erlenmeyer flask. The mixture was heating in a microwave at 450W for 5 minutes to completely dissolve the agarose. After cooling the mixture to ±60°C (under running tap water) 15 µl of ethidium bromide (500µg/ml) was added to the gel mix. Ethidium bromide is a potent mutagen that intercalates between the nucleotide bases of DNA and fluoresces following stimulation with ultraviolet light (280nm). The fluorescence of ethidium bromide therefore is used as a marker to both visualise DNA in the gel, and following capture with photographic film, to determine the expression ratio for semi-quantitative analysis. The gel mix was then poured into the gel dye and three sixteen sample gel combs were set. The gel was left to cool and set for a minimum of 30 minutes. The gel was transferred to the gel tank and submerged under 0.5x TBE running buffer. 10 µl of the PCR product was mixed with 2 µl of a 6x sample loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% cyanin orange in 0.5x TBE) and the whole 12 µl was loaded into the well. The glycerol in the loading buffer increased the density of the DNA sample which prevents the DNA from "floating" out of the well during loading. Bromophenol blue (BMB) and cyanin orange (CO) are tracking dyes that give an indication of the distance migrated by the DNA during electrophoresis. The samples were loaded in sequence as determined by their location in the original vein (proximal to distal), for each individual primer set.

The positive and negative controls as well as a 100bp DNA ladder (Sigma) were run simultaneously with each sample set. The DNA fragments were resolved by applying an electric field of 120mV, 60mA for 35 minutes. On completion, the gel was viewed on an UltraViolet transilluminator (UV Ltd, UK). The product size was estimated from the migration of the 100bp ladder and compared to the theoretical size of the product. Analysis of the controls (single band in positive, no band in negative) indicated a successful PCR. Finally a photograph of the gel, with all primer sets was taken for scanning densitometry analysis using an exposure of ½ second with the F stop set to 5.8.

2.4 ANALYSIS OF PROTEIN EXPRESSION

2.4.1 Background

Immunoassays are routinely used to measure protein in complex mixtures due to their specificity and high sensitivity. Here, two distinct assays have been utilised to measure protein, namely enzyme linked immunoassay (ELISA) and polyacrylamide gel eletrophoresis (PAGE) with Western Blotting. Analytic separation of protein is commonly investigated using PAGE under conditions that prevent aggregation and ensure complete dissociation of protein into individual polypeptide subunits (reducing SDS-PAGE). The separated proteins can then either be simply stained in the gel or transferred onto a solid support in such a manner that the pattern of the proteins electrophoretic separation is maintained. The proteins on the support are probed with antibodies raised against a specific antigenic epitope, to detect the protein of interest (Western Blotting). With ELISA the protein of interest is captured by an antibody that itself is immobilised to a solid support (96 well plate). Contaminating cellular constituents are then discarded and the captured ligand is detected with another antibody conjugated to a reporter system. ELISA and SDS-PAGE/western blotting are sensitive analytically techniques which can readily discriminate a specific protein from a complex protein mix, for the analysis of protein expression.

2.4.2 Sandwich ELISA

ELISA assays can be separated into four different varieties dependant on generally the manner with which the antigen of interest is presented. i) Direct ELISA involves attachment of the antigen/crude lysate directly to the solid support. The ligand is then detected directly with an enzyme-labelled antibody. Direct ELISA generally makes measurement of crude samples difficult since contaminating proteins compete for

plastic binding sites and they are not very sensitive. ii) Similarly, Indirect ELISA also involves attachment of antigen to the solid phase followed by the detection of antigen with an antibody. However the primary antibody itself is not labelled but is detected by a second antibody (raised against the first) that is conjugated to an enzyme label. This format increases the sensitivity of the assay compared to Direct ELISA and is generally used for the detection of specific antibodies in sera. iii) A third type of ELISA is the Competition Assay, which involves the simultaneous addition of 'competing' antibodies or proteins. The decrease in signal in samples where the second antibody or protein is added gives a highly specific result. iv) The final assay and most popular is the Sandwich ELISA. Sandwich ELISA involves the attachment of a capture antibody to a solid phase support. Samples containing known or unknown antigen are then added in a buffer that will minimize attachment to the solid phase (96 well plate) while facilitating formation of antibody antigen binding. The lysate is discarded and the captured antigens are detected with a second antibody (enzyme labelled) raised against the same antigen. The Sandwich ELISA is the method which gives the most specific and sensitive results for cytokine antigens found in very low concentrations.

2.4.3 **Protocol: Quantikine™ human sVEGFR1 ELISA**

Levels of plasma s.flt-1 (soluble VEGFR1) were determined using a quantitative sandwich enzyme immunoassay kit (Quantikine™ human sVEGFR1; R&D Sytems, UK) according to the manufacturer's instructions. Briefly, 100 µl of Assay Diluent was added to each well of the 96-well polystyrene microplate, provided pre-coated with a mouse monoclonal antibody against VEGFR1. 100 µl of either recombinant human s.flt-1standard (doubling dilutions 31.25 – 2000 pg/ml), or plasma sample was

added to each well in duplicate. The plates were covered with the adhesive strip provided and incubated on a horizontal orbital shaker at 500rpm for 2 hours at room temperature. Following incubation, plates were aspirated and washed (x4) using 400 µl wash buffer per well. 200 µl of detection antibody (polyclonal antibody against VEGFR1 conjugated to horseradish peroxidase) were added to each well. The plates were covered (as above) and incubated on the orbital shaker for 2 hours at room temperate. Following washing (x 4) 200 µl of Substrate Solution was added to each well. The plates were wrapped in aluminium foil to protect from light and incubated for 30 minutes at room temperature. 50 µl of Stop Solution was added to each well and the optical density (OD) at 450nm (correction at 540nm) was recorded (Dynex microplate reader). For analysis, the mean of duplicate experiments (each with duplicate wells/sample) was calculated and the quantity of s.flt-1 in each sample determined by extrapolation from the standard curve.

2.4.4 **Polyacrylamide gel electrophoresis**

In solution proteins are amphoteric and have a net charge at any pH other than their isoelectric point. As discussed previously (chapter 2.3.5) electrophoresis describes the situation in which a charged molecule in solution is separated in an electric field at constant viscosity and applied voltage. Electrophoretic mobility therefore can be used to resolve proteins in two fundamentally different ways, either by using their intrinsic charge differences (isoelectric focusing) or by imparting a large extrinsic charge on the protein (SDS-PAGE). Before gel systems were developed protein electrophoresis was performed in the Tiselius free boundary system (Scopes 1993). Dissolved protein mixtures were placed in a U shaped buffer filled channel and exposed to an electrical field. Resolution was poor however and any disturbance of

the apparatus compromised the separation. Gel matrices were developed to serve as solid supports during electrophoresis to stabilise the separated components by minimising movement through diffusion and facilitating easy handling of the separated protein. Although starch was the first matrix used successfully the development of the synthetic gel medium polyacrylamide, improved resolution dramatically (Ornstein *et al.,* 1964). Further developments which improved the resolving power of polyacrylamide gel electrophoresis included the use of a stacking gel with discontinuous buffer systems (Ornstein *et al.,* 1964) and the incorporation of the detergent, sodium dodecyl sulphate (SDS) into the buffers (Laemmli 1970).

In the Laemmli system, the strong anionic detergent SDS is used in combination with reducing agents and heat, to totally denature the protein before the sample is loaded on the gel. SDS binds strongly to proteins reaching saturation at approximately 0.1% SDS (1 detergent molecule per 2 amino acid residues, Reynolds *et al.,* 1970). The hydrophobic tail of SDS interacts with the hydrophobic domain of the denatured polypeptide which confers a strong extrinsic negative charge to the polypeptide. As SDS binds protein in the constant ratio (1.4g detergent per gram of polypeptide) polypeptides of different size will bind an amount of SDS that is in proportion to their size. The amount of SDS bound by the polypeptide is therefore, almost always directly proportional to the molecular weight of the polypeptide irrespective of its specific amino acid sequence. Consequently the charge to mass ratio (***Q*/r**) for all polypeptides in solution with saturating SDS is virtually identical and the separation of the SDS-polypeptide complexes occurs according to size as a result of the molecular sieving capability of the gel.

In most cases, SDS-PAGE is performed using the discontinuous buffer system described by Ornstein and Davies. Here the sample and running buffer have a different pH and ionic strength to the buffer used to cast the gel. The sample and stacking gel contain Tris-HCl at pH 6.8 the resolving gel contains Tris-HCl at pH 8.8, while the running buffer is comprised of Tris-Glycine at pH 8.3. All the components contain SDS at a minimum 0.1% (Laemmli 1970). During electrophoresis, the chloride ions in the sample and stacking gel buffer form the leading edge of the moving boundary, while the slower moving glycine forms the trailing edge. Between the leading and trailing edge is a zone of low conductivity and steep voltage gradient. This sweeps the SDS-polypeptide complexes through a stacking gel of high porosity and deposits the complexes in a thin zone on the surface of the resolving gel. The higher pH of the resolving gel initiates the ionisation of glycine which increases the rate of migration of the anion. The glycine anions quickly migrate through the stacked polypeptides catching up with and migrating immediately behind the chloride ions in the resolving gel. Expelled from the moving boundary, the SDS-polypeptide complexes migrate through the resolving gel in a zone of uniform voltage and pH and are separated according to size by the molecular sieving effect of the gel matrix.

The molecular sieving properties (effective range of separation) of a SDS-PAGE gel are determined by the size of the pores in the gel. Polyacrylamide gels are composed of chains of polymerised acrylamide crosslinked with N,N'-methylenebisacrylamide to form a gel of uniform pore size. The pore size is dependant on both the concentration of acrylamide used and the degree of crosslinking in the gel. The porosity of the gel decreases as the bisacrylamide:acrylamide ratio increases reaching a minimum at a molar ratio of approximately 1:20. For consistency, a standard

bisacrylamide:acrylamide molar ratio of 1:29 is commonly used in SDS-PAGE and pore size is changed simply by varying the concentration of acrylamide.

Today almost all analytical electrophoresis of protein is undertaken with SDS-PAGE. The advantages of SDS-PAGE are the ease of operation, the method is relatively quick, has a high level of sensitivity and yields sharp resolution of complex protein mixtures into their individual components. SDS-PAGE enjoys wide spread application in protein isolation and characterisation protocols including the separation of protein mixtures and measurement of the relative abundance of a protein in a sample, determination of approximate molecular weight of a polypeptide and analysis of purity of protein isolation.

Here we have used the NuPAGE® Novex Bis-Tris Electrophoresis System from Invitrogen. NuPAGE® Bis-Tris is a discontinuous SDS-PAGE, pre-cast polyacrylamide mini-gel system which unlike the Laemmli system is maintained at a neutral pH (pH 7.0). Similar to Ornstein and Davies system, chloride supplied by the gel buffer (Bis-Tris^{+} and Cl^{-}, pH 6.4) is the leading ion. MOPS-SDS running buffer, however has a trailing ion in neutral pH (running buffer Tris^{+}, MOPS^{-} and dodecyl sulphate^{-}, pH 7.3-7.7). The combination of a lower gel buffer and running buffer pH results in a significantly lower operating pH during electrophoresis contributing to increased stability of both the polyacrylamide gel matrix and protein sample (reduced deamination and alkylation reactions) which results in better protein band resolution.

2.4.5 **Protocol: Denaturing NuPAGE® Bis-Tris electrophoresis**

For each sample, 20 µg total protein was added to NuPAGE® LDS sample buffer and NuPAGE reducing agent (500mM dithiothreitol, DTT) as indicated in (Table 2.6)**.** Samples were heated at 70°C for 10 minutes and transferred to and stored on ice. Prestained and biotinylated broad range protein markers (NEB Ltd, UK) and control protein (ECV304, microvascular endothelial cell lysate) were similarly prepared. One litre of NuPAGE® MOPS-SDS running buffer (1x) was prepared from stock (20x) and mixed thoroughly (Appendix 4). A NuPAGE® Novex gradient gel (4-12% acrylamide) was removed from its pouch, rinsed with distilled water (x3) and the well-comb carefully removed. The wells were rinsed with 200 µl of running buffer (x3) and after removal of the tape from the bottom of the cassette, the gel was placed in the Mini-Cell module (wells facing inwards toward the Buffer Core). Finally the gel cassette was locked in place with the Gel Tension Wedge. The upper buffer chamber (cathode) was filled with 200 ml MOPS-SDS running buffer containing 500 µl NuPAGE® Antioxidant (proprietary reagent) and checked for leaks. NuPAGE® Antioxidant co-migrates with protein during electrophoresis which maintains the proteins in a reduced state and protecting sensitive amino acids such as methionine and tryptophan from oxidation (NuPAGE® Technical Guide). After the loading of protein samples, marker and control the lower buffer chamber (anode) was filled with 600 ml MOPS-SDS running buffer and the proteins were separated at a constant voltage of 200 volts (current: start 100-115mA, end 60-70mA) for 50 minutes. On completion the gel cassette was removed from the Mini-Cell module, cracked opened with a gel knife and the NuPAGE® gel prepared for western blot transfer.

2.4.6 **Western blot**

Western blotting is a sensitive technique that exploits the specific recognition of an antibody for its antigen. In western blotting, protein is transferred from acrylamide gel and immobilised on a membrane support in such a manner that the pattern of the protein's electrophoretic separation is maintained. The membrane is probed with antibodies raised against the specific protein of interest and bound antibody/antigen complex is detected with labelled secondary antibody following the addition of a detection substrate. Protein was transferred from NuPAGE® Novex gel with the XCell II™ Blot Module (Invitrogen Ltd, UK) and protein-antibody complexes were detected by chemiluminesence with Phototype® -HRP western blotting detection system (New England Biolabs Ltd, UK). The Phototype® -HRP western blotting detection system is an extremely sensitive (10^{-12}g) non-radioactive method to detect immobilised antigens using antibodies labelled with horseradish peroxidase (HRP) and the chemiluminescent substrate Luminol®. In the presence of hydrogen peroxide HRP converts Luminal® to an excited intermediate dianion. The dianion emits light on return to its ground state which is captured on X-ray film for analysis.

2.4.7 **Protocol: XCell II™ Blot Module protein transfer**

One litre of NuPAGE® Transfer Buffer (1x) was prepared from stock (20x) mixed thoroughly and NuPAGE® Antioxidant and methanol was added (Table 2.7). [Methanol increases the hydrophobic interaction between protein and membrane required for binding. The volume of methanol added therefore is determined by the number of gels transferred (10% per gel)]. Gloves were worn for all manipulations of polyvinylidene fluoride (PDVF) membranes. A pre-cut NuPAGE® PDVF membrane was wet with methanol for 30 seconds, rinsed in water for 1 minute (x3) and

incubated together with blotting pads and filter paper for at least 20 minutes before use. The blot module was assembled by first placing two blotting pads on the bottom (cathode) as shown. The NuPAGE® gel removed from the cassette was carefully overlaid with a piece of saturated filter paper, all air bubbles were excluded and rapidly inverted to capture the gel on the filter paper. The filter paper-NuPAGE® gel was then placed on top of the blotting pads (filter paper down). The pre-cut membrane was carefully aligned on top of the gel followed by the last piece of filter paper and two blotting pads ensuring that no air bubbles were trapped between the layers. The assembly was checked to ensure there was no contact between the paper layers, the top (anode) was attached and the XCell II™ Blot Module placed in the Mini Cell unit. The blot module was filled with NuPAGE® Transfer Buffer just covering the gel-membrane assembly, while the outer chamber was filled with 650 ml cold water. Protein was transferred at a constant voltage of 30 volts (current: start 170mA, end 110mA) for 90 minutes.

Reagent	Volume (µl)
sample	x
NuPAGE® LDS sample buffer (4x)	2.5
NuPage® reducing agent (10x)	1
deionised water	to 6.5
Total volume	**10**

Table 2.6 Sample preparation for NuPAGE® Bis-Tris electrophoresis (Appendix 4)

Reagent	1 gel (ml)	2 gels (ml)
NuPAGE® Transfer Buffer (20x)	50	50
NuPAGE® Antioxidant	1	1
Methanol	100	200
water	849	749
Total volume	**1000**	**1000**

Table 2.7 Preparation of NuPAGE® Transfer Buffer (Appendix 4)

2.4.8 **Protocol: Phototype®-HRP chemiluminescence detection**

The electroblotter was dismantled, the membrane washed with Tris buffered saline (TBS, 20 mM Tris base pH 7.4, 137 mM NaCl) and transferred to a rocking platform. Blots were first stained for total protein with Ponceau S (Sigma-Aldrich Ltd, UK) to confirm equal protein loading before proceeding with antibody detection. Non specific binding sites on the membrane were blocked with Block buffer (TBS, 10% Marvel, 0.1% Tween 20) for one hour at room temperature. Block buffer was discarded and the membrane washed twice with wash buffer (TBS, 01% Tween 20). The membrane was incubated with a rabbit anti-human β-catenin polyclonal antibody diluted as per the manufacturers recommendation (1:2000, # 9562, Cell signalling Technology™, UK) in diluent (TBS, 5% Marvel, 0.1% Tween 20) with gentle agitation overnight at 4°C. Primary antibody diluent was discarded and the membrane washed three times with wash buffer. Bound antibody was detected with a goat anti-rabbit antibody conjugated to HRP (1:5000 fold dilution) with gentle agitation for one hour at room temperature. The detection antibody was discarded, the membrane washed five times with wash buffer and transferred to Sarafan film on the bench for processing. The LumiGLO chemiluminesence substrate and hydrogen peroxide (both 20x) were diluted in 10ml and incubated with the membrane for 1 minute in the darkroom. Excess solution was discarded, the membrane enclosed completely in Sarafan film and exposed to Hyperfilm™ ECL (Amersham Biosciences Ltd) for 5 minutes. The film was developed manually with Kodack BioMax reagents (Sigma-Aldrich UK). The integrated optical density (IOD) of each band was measured by scanning densitometry (LabWorks Imaging and Acquisition software, UVP, Cambridge UK), and normalised for protein loading against IOD values from the Ponceau S membrane stain.

2.5 SCANNING DENSITOMETRY

2.5.1 Background

Scanning densitometry is a software package used for the quantitative measurement of the signal intensity from digitalised images. The shade of each pixel in the area of interest (AOI) on the gel image is characterised according to a greyscale range comprising 256 different shades. Discrimination of the differences between the intensity of different pixel shade facilitates the quantification of signal intensity of each pixel used to construct the band in the AOI. This data is converted to a lane profile graph which displays either the optical density (X-ray and gel image) or intensity (fluorescent light image) for each band under investigation. The band is represented by a peak in the profile graph which represents the molecular weight value for that particular band, relative to the molecular weight marker used. The area under the peak is used to calculate the volume (amount) of signal in each band and is termed the Integrated Optical Density (IOD). With IOD the relative abundance of a PCR product (agarose gel) or protein (Western Blot) can therefore be determined from the band intensity due to the captured fluorescence or chemiluminescence in the original image

2.5.2 Analysis of DNA agarose gels

For reproducibility, all agarose gels were photographed with a Kodak Polaroid camera using the default settings (exposure1/2 second, F-stop 5.6-8). The photograph was digitalised with an Epson GT-9500 scanner at a pixel depth of 800 with a 150% enlargement. All digital gel images were analysed at a single sitting with Labworks™ Image Aquisition and Analysis software (Ultra-Violet Products Ltd). Analysis of the image was performed with the 1D-Gels tool palette using the same

parameters for all gels analysed. The gel was imported into Labworks™ and the 1D-Gel tool palette was activated. Lanes of 200 pixels wide were assigned to each lane investigated and the bands (AOI) marked automatically. Analysis of the lane profile graph was examined for each lane and spurious banding was deleted. No background correction was used and the default was the greyscale range as determined from the image under investigation. The relative abundance of each DNA band in the image, as determined from band fluorescence was used to calculate the IOD. Semi-quantitative analysis was performed by comparing the IOD of the band of interest to the IOD of the standard, here GAP3DH (Figure 2.5).

2.5.3 **Analysis of western blots**

The developed film of the western blot was digitalised with an Epson GT-9500 scanner at a pixel depth of 800 with a 150% enlargement. All digital western blot images were analysed at a single sitting with Labworks™ Image Aquisition and Analysis software as described. The integrated optical density (IOD) of each band was measured reported normalised for protein loading against IOD values from Ponceau S membrane stain (Figure 2.6).

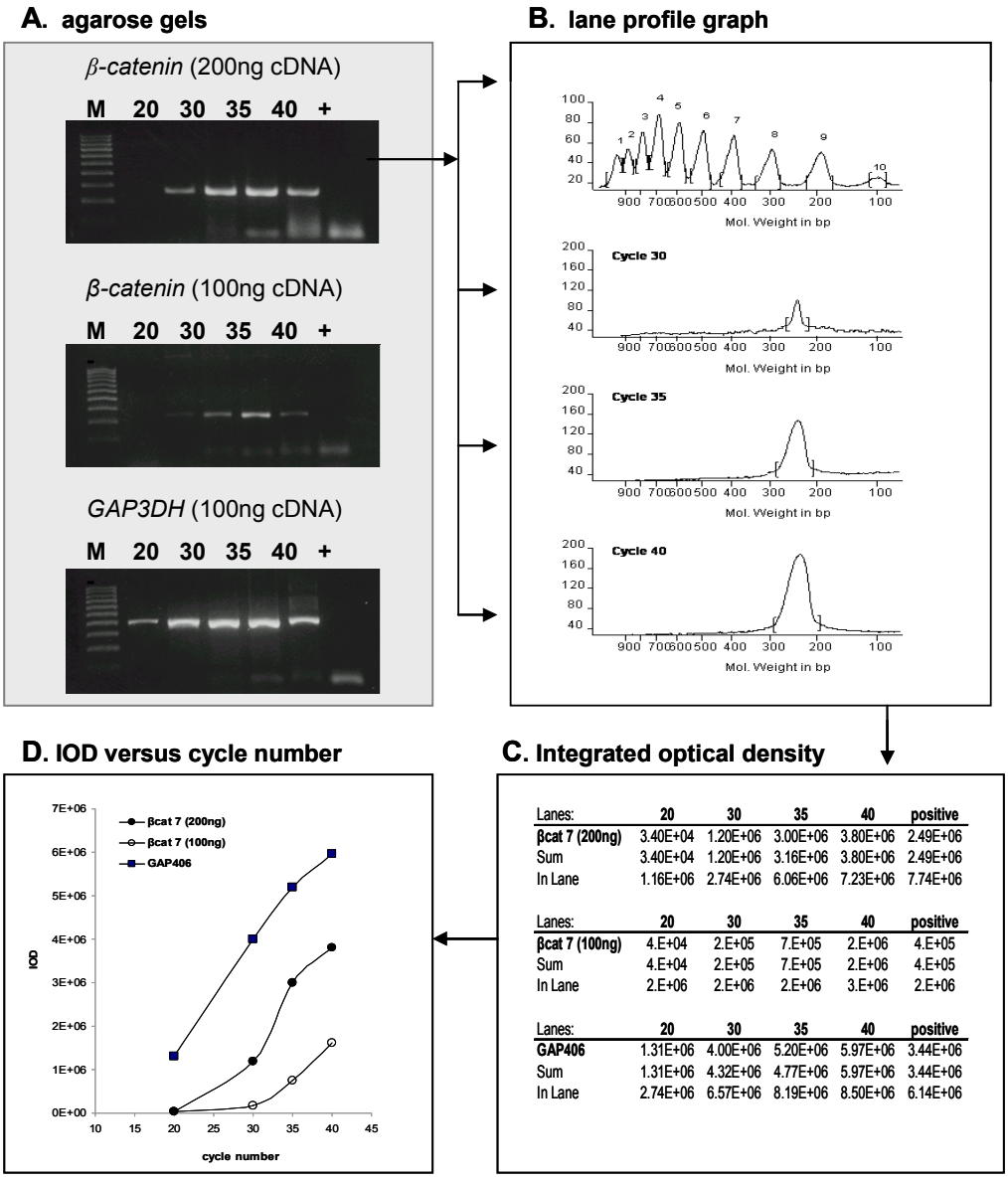


Figure 2.5 Scanning densitometry analysis of PCR products.

Here the kinetics of the PCR reaction for the primer sets (BCAT 7 and GAP406) were investigated to determine optimal cycle number. All reactions contained 2 mM $MgCl_2$ but had differing amounts of cDNA template (200ng or 100ng for BCAT 7 and 100ng for GAP406). PCR products, separated by DNA agarose gel electrophoresis are capture on film and digitalised (**A**). For each product band, ethidium bromide fluorescence is captured as a lane profile by scanning densitometry (**B**), and recorded as an Intergrated Optical Density (IOD) (**C**). Plot of IOD versus cycle number indicates the linearity of the reaction per starting quantity cDNA, for each primer pair investigated (**D**).

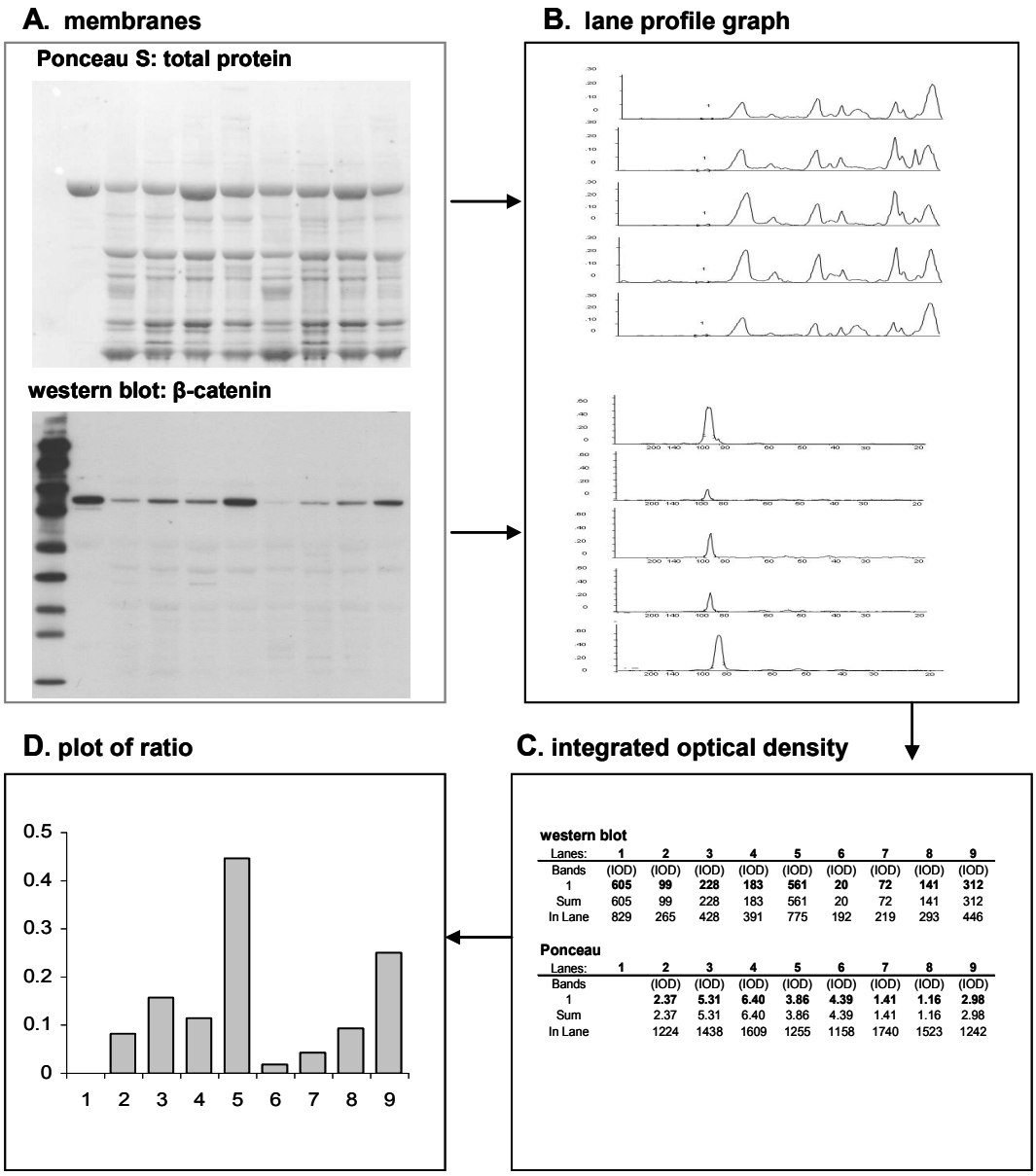


Figure 2.6 Scanning densitometry analysis of western blot.

Total protein, separated by SDS-PAGE was transferred to membranes, stained with Ponceau S and probed with anti β-catenin antibody (**A**). Each product band is captured as a lane profile by scanning densitometry (**B**), and recorded as an Intergrated Optical Density (IOD) (**C**). Values reported are IOD ratio of β-catenin divided by total protein Ponceau-S (**D**).

2.6 STATISTICS

The age of individuals within each group are presented as a median value with range in parenthesis, and significance between groups evaluated by Mann Whitney U test. The distribution of experiment results obtained for gene transcription and protein expression were tested for normality with Kolmogorov-Smirnov test and descriptive statistics examining skewness and kurtosis. Comparisons between the experimental groups were investigated with Students T-test (paired or unpaired), analysis of variance (ANOVA) or Kruskal-Wallis analysis of variance (ANOVA) and presented as mean (± s.e.m.) or median (with interquartile range) as appropriate. Comparisons of differences in gene transcription in vein sections per position, from proximal to distal were made by one-way ANOVA with Bonferroni's correction to identify differences between individual group means. Also, to examine for possible correlations between position and levels of gene transcription or protein expression, results were analysed by Kendall's bivariate correlation. All tests (where appropriate) were two-tailed; statistical significance was accepted at $p<0.05$; analyses yielding non-statistically significant results are cited as $p>0.1$; actual p values are reported to three decimal places to $p<0.001$. All statistical analysis reported in this study was investigated with the software package SPSS version 11.5 (IBM).

2.6.1 Transcription of *VEGF*-A and its receptors

Ages of individuals, per group, are presented as median values with range in parenthesis, and comparisons between groups were made by Mann & Whitney U test, two-tailed. Experimentally-obtained results for levels of gene transcription were of a similar and normal-shaped distribution, as determined by a Kolmogorov-Smirnov one-sample test of normality, and descriptive exploration and analysis of Skewness,

and are presented as mean (s.e.m.) values. Comparisons of the levels of gene transcription between experimental groups were made by one-way or Kruskal-Wallis analysis of variance (ANOVA). Comparisons of differences in gene transcription in vein sections per position, from proximal to distal (see Figure 2.1), were made by one-way ANOVA with Bonferroni's correction to identify differences between individual group means. Also, to examine for possible correlations between position (proximal – distal; sections 1 – 5) and levels of gene transcription, results were analysed by Kendall's bivariate correlation analysis. All tests (where appropriate) were two-tailed; $p<0.05$ was considered to be statistically significant; actual p values are reported to three decimal places to $p<0.001$.

2.6.2 **Release of s.flt-1**

The overall ages of individuals within each subject group are presented as a median, with range in parenthesis and comparisons of experimental groups made by Mann & Whitney 'U' test. Experimentally obtained results for the levels of plasma s.flt-1 were of a uniform, but not normal, distribution as determined by descriptive statistics examining skewness and kurtosis, and have been presented as (median values; range). Graphical presentation of results is by box-plot (median, inter-quartile range, and range). Comparison of the levels of plasma s.flt-1 between controls and subjects, and changes in plasma s.flt-1 before and after cuff application (per group) were analysed by Kruskal-Wallis analysis of variance. Percentage changes in plasma s.flt-1 after cuff application were determined by '(after cuff – before cuff) / (before cuff) x 100' and are presented as a mean (± s.e.m.) percentage change. Statistical significance was accepted at $p<0.05$ and reported significant to $p<0.001$ level, while non-statistical significance was reported as $p>0.1$.

2.6.3 *β-catenin*, *c-myc* and *cyclin D1* transcription and expression

Age of individuals are presented as a median value with range in parenthesis, and significance between groups evaluated by Mann & Whitney 'U' test. Experimentally obtained results for *β-catenin*, *c-myc* and *cyclin D1* transcription and β-catenin protein expression are presented as median values with interquartile range in parenthesis following analysis by Kolmogorov-Smirnov one sample test of normality and analysis of Skewness. Comparisons between levels of *β-catenin*, *c-myc* and *cyclin D1* transcription and β-catenin protein expression between groups were made by Kruskal-Wallis analysis of variance. Possible correlations between factors were examined using Kendall's bivariate correlation analysis. All tests (where appropriate) were two tailed. Statistical significance was accepted at $p<0.05$, with actual p values reported significant to three decimal places to $p<0.001$.

Chapter 3

RESULTS

3.1 **TRANSCRIPTION OF *VEGF-A* AND ITS RECEPTORS**

3.1.1 **Background**

The homeostatic response of the vein wall to environmental stimuli like a change of luminal pressure, disturbed blood flow, venous stasis and hypoxia should be the production and release of agents that mediate vessel wall reactivity (Luscher 1991; Micheils *et al.,* 1997; Browse *et al.,* 1999). In normal vein, venous stasis and disturbances in blood flow induce a rapid change in the level of detectable VEGF-A (Hollingsworth *et al.,* 2001A), the generation of which is regulated at a number of levels to modulate VEGF-A protein availability and activity (see chapter 1.2.2). As the control of transcription of VEGF-A (and its receptors) involved in maintaining vascular reactivity might become altered, either as a causative mechanism, or simply as a result of the pathological process itself, examination of such genes may help to elucidate the mechanism(s) underlying the development of VVs.

3.1.2 **Aim**

To examine the pattern of transcription of genes for *VEGF-A* (the soluble isoforms *VEGF-A_{121}* and *VEGF-A_{165}*) and its receptors *VEGFR1* (and *s.flt-1*) and *VEGFR2* in the wall of the GSV, and in relation to the underlying venous incompetence in primary VVs.

3.1.3 **Methods**

This study was conducted with approval of the Joint UCL/UCLH Committees on the Ethics of Human Research as described in chapter 2.1.1. With informed consent, samples of varicose GSV were obtained from patients undergoing sapheno-femoral ligation and stripping of the long saphenous for the treatment of primary VVs. Control GSV was obtained from patients undergoing cardiac bypass, and who had no

clinical evident symptoms of varicose disease in either limb (see chapter 2.1.2). Samples of varicose or control GSV collected on ice at operation were processed as described in chapter 2.1.2. Total RNA was extracted with TRI Reagent™ (see chapter 2.2.2) and then reverse transcribed to cDNA as described in chapter 2.3.1.

The gene activity of the soluble *VEGF* isoforms (*VEGF-A*$_{121}$ and *VEGF-A*$_{165}$) and the VEGF receptors (*VEGFR1*, *s.flt-1* and *VEGFR2*) were examined by semi-quantitative PCR (see chapter 2.3.4). Gene products were fractionated by agarose gel electrophoresis (see chapter 2.3.6 and representative gel pictures Figure 3.1-3) and analysed by scanning densitometry as described in chapter 2.5.2. The author thanks Mr Cooper for the initial round of RT-PCR investigations used in this study.

Statistical analysis of results was undertaken as described in chapter 2.6.1.

A.

Control					
SFJ status	competent				
segment status	c	c	c	c	c
position					

B.

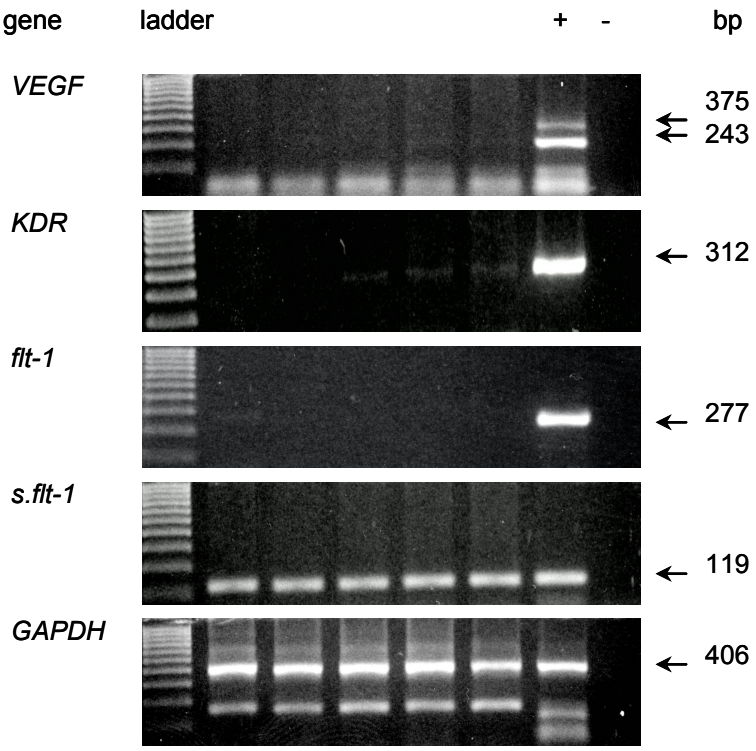


Figure 3.1 RT-PCR gel analysis of *VEGF-A* and its receptors in control vein.
For each individual segment analysed, the competency of the SFJ and vessel wall was determined from duplex scan and classified as being competent with no reflux (c) or incompetent with reflux evident (i) (**A**). Gene transcription was examined by semi-quantitative RT-PCR. Products were visualised by agarose electrophoresis and the IOD of each band measured by scanning densitometry. Levels of gene transcription were reported as IOD ratio of gene PCR product normalised against control house keeping gene *GAP-3* (*GAPDH*) (**B**).

A. **Competent**

SFJ status	competent				
segment status	c	c	i	i	i
position	Px	PM	Mid	MD	Dis

B.

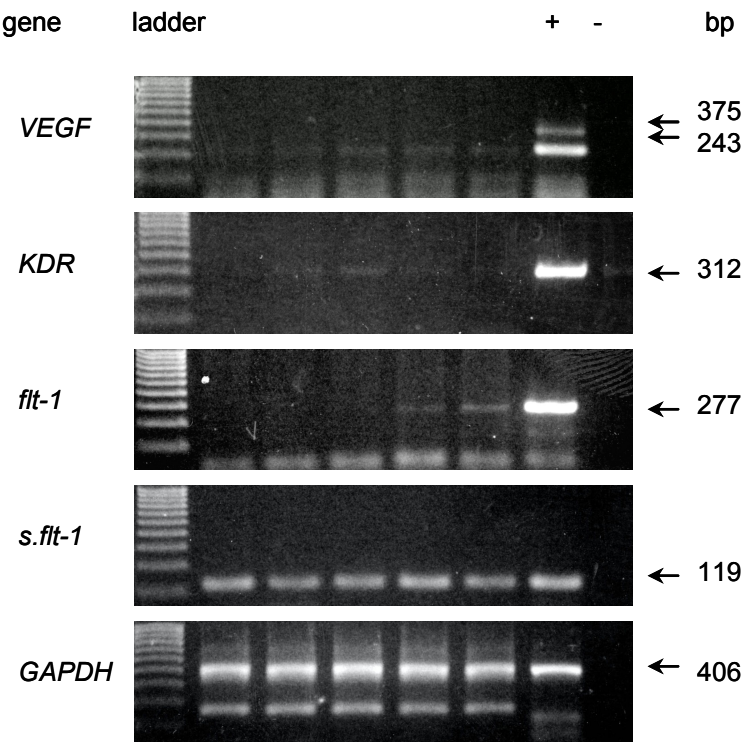


Figure 3.2 RT-PCR gel analysis of VVs segments with a competent SFJ.
For each individual segment analysed, the competency of the SFJ and vessel wall was determined from duplex scan and classified as being competent with no reflux (c) or incompetent with reflux evident (i) (**A**). Gene transcription was examined by semi-quantitative RT-PCR. Products were visualised by agarose electrophoresis and the IOD of each band measured by scanning densitometry. Levels of gene transcription were reported as IOD ratio of gene PCR product normalised against control house keeping gene *GAP-3* (*GAPDH*) (**B**).

A.

Incompetent					
SFJ status	incompetent				
segment status	i	i	i	i	i
position	Px	PM	Mid	MD	Dis

B.

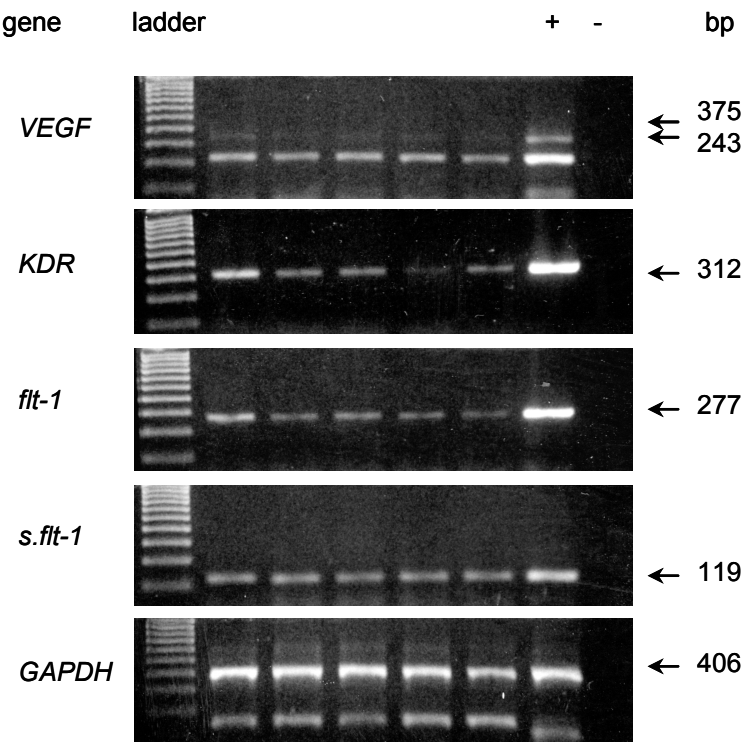


Figure 3.3 RT-PCR gel analysis in VVs with an incompetent SFJ.

For each individual segment analysed, the competency of the SFJ and vessel wall was determined from duplex scan and classified as being competent with no reflux (c) or incompetent with reflux evident (i) (**A**). Gene transcription was examined by semi-quantitative RT-PCR. Products were visualised by agarose electrophoresis and the IOD of each band measured by scanning densitometry. Levels of gene transcription were reported as IOD ratio of gene PCR product normalised against control house keeping gene *GAP-3* (*GAPDH*) (**B**).

3.1.4 **Results**

Patient demographics: GSV was obtained from 23 individuals resulting in a total of 97 samples overall available for gene analysis (Table 3.1). Of the 23 individuals examined, 18 had primary VVs as confirmed by venous duplex scan resulting in a total of 83 samples available for gene analysis. Of these, 7 individuals had a functionally intact and competent SFJ (29 samples; 4 male, 3 female) while for the remaining 11, the SFJ was incompetent (54 samples; 3 male, 8 female). Control GSV was obtained from 5 individuals (3 male, 2 female) resulting in a total of 14 control samples available for analysis.

Overall, patients with VVs were younger than those from whom control veins were taken ($p<0.030$, Mann-Whitney U, Table 3.1). [This age difference was mainly accountable by the larger subgroup of female individuals with an incompetent SFJ, whose median age was less than those with VVs with a competent SFJ]. Individuals with an intact and functional SFJ had a greater, although not statistically significant median age than those individuals who presented with an incompetent SFJ with blood reflux.

RNA of suitable quality was isolated from all vein sections analysed as illustrated in representative gels for individuals from control (Figure 3.1), competent SFJ (Figure 3.2) and those with an incompetent SFJ (Figure 3.3).

Description	Varicose			Control
	competent SFJ	incompetent SFJ	total	
Individuals, *n* =	**7**	**11**	**18**	**5**
median age (range)	62 (28-69)	43 (27-58)	48 (27-69)*	63 (50-72)
male, *n* =	4	3	7	3
female, *n*=	3	8	11	2
mRNA samples, *n* =	**29**	**54**	**83**	**14**
male, *n* =	15	15	30	7
female, *n* =	14	39	53	7

Table 3.1 Patient demographics and samples for analysis of gene transcription.
Vein samples were obtained from 23 individuals. 18 patients had primary VVs (7 male, 11 female) resulting in 83 samples for analysis. Control GSV was obtained from 5 individuals (3 male, 2 female) resulting in a total of 14 control samples available for analysis. Overall, patients with VVs were younger than those from whom control veins were taken ($p<0.030$, Mann-Whitney U). SFJ, sapheno-femoral junction, *$p<0.03$ versus control vein, Mann & Whitney U

Varicose and control veins overall: For VVs overall, the transcription of all genes except *s.flt-1* were elevated in sections of VVs compared to those from control veins (Figure 3.4); *VEGF-A*$_{121}$, *VEGF-A*$_{165}$ and *VEGFR*2 (all, p<0.001), *VEGFR1* (p<0.002); *s.flt-1* (p=0.743); all Kruskal-Wallis.

Competence of the SFJ: Results from VVs were sub-divided and analysed specifically in conjunction with competence at the SFJ. Individuals in whom the SFJ was intact and functional (as determined from duplex scan) had a greater, but not statistically significant, median age than those in whom the SFJ was incompetent and showing reflux (Table 3.1). In VVs with a functional SFJ, transcription of *VEGF-A*$_{165}$, *VEGFR2*, *VEGFR1* and *s.flt-1* was similar to that of control veins (Figure 3.5). However, transcription of the *VEGF-A*$_{121}$ isoform was elevated (p<0.02; see Figure 3.5), although not to the levels seen when the SFJ was incompetent.

In contrast, when the SFJ was incompetent the levels of transcription of *VEGF-A*$_{121}$, *VEGF-A*$_{165}$, *VEGFR2*, and *VEGFR1* were elevated compared to either those where the SFJ was functional (all p<0.001), or to control veins (all p<0.001, see Figure 3.5).

Competence of the vein wall: To examine levels of gene transcription in conjunction with vein competence, results were now grouped and analysed as follows. For each individual VVs sample analysed, the original position of the segment was cross referenced to the duplex scan, and the vessel wall was classified as being (i) competent with no reflux, or (ii) incompetent with evident reflux. Pre-operative duplex scans on control individuals used in this study indicated that the vessel wall of all control vein samples used were indeed competent.

In VVs, competent sections had elevated levels of transcription of *VEGF-A*$_{121}$ (p<0.02), *VEGF-A*$_{165}$ (p<0.006), and *VEGFR2* (p<0.007) when compared to control vein (Figure 3.6). *VEGFR*1 transcription was also elevated, although not significantly compared to control and as previously there was no change in the transcription of *s.flt-1*. In incompetent sections the levels of transcription for all genes except *s.flt-1* were elevated compared to sections from control veins (*VEGF-A*$_{121}$, *VEGF-A*$_{165}$, *VEGFR2* and *VEGFR1*, all p<0.001, Figure 3.6).

Interestingly transcription of *VEGF-A*$_{165}$ and the receptors *VEGFR2*, *VEGFR1* and *s.flt-1* were similarly elevated in competent and incompetent VVs sections. Furthermore, there was a trend for greater transcription of *VEGF-A*$_{121}$ in incompetent sections of VVs as opposed to competent VVs sections (p=0.074; see Figure 3.6).

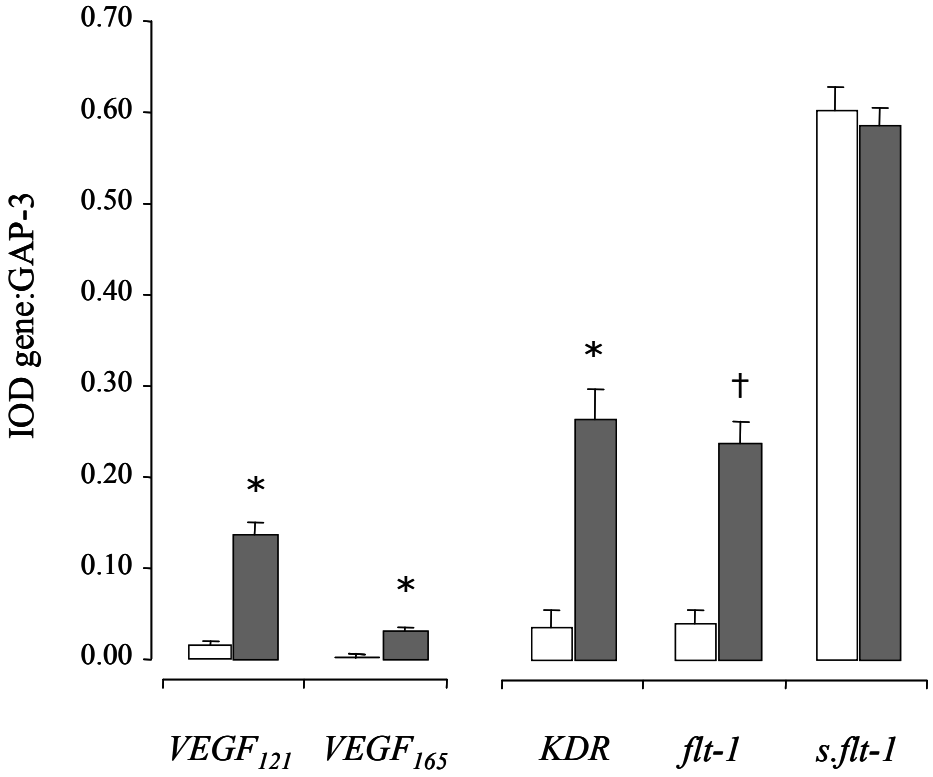


Figure 3.4 Transcription of *VEGF-A* and its receptors in VVs overall.

Control (open bars) and VVs (grey bars). IOD gene:GAP-3 is the ratio of the integrated optical density (IOD) of the specific gene product to its' corresponding product for *GAP-3* as determined by scanning densitometry. Bars represent means values, and error-bars are s.e.m. (n=83 sections analysed from VVs; n=14 sections analysed from control vein sections; all with two PCR analyses per section for each gene examined). *p<0.001 and $^{\dagger}p$<0.002 versus control vein, Kruskal-Wallis test.

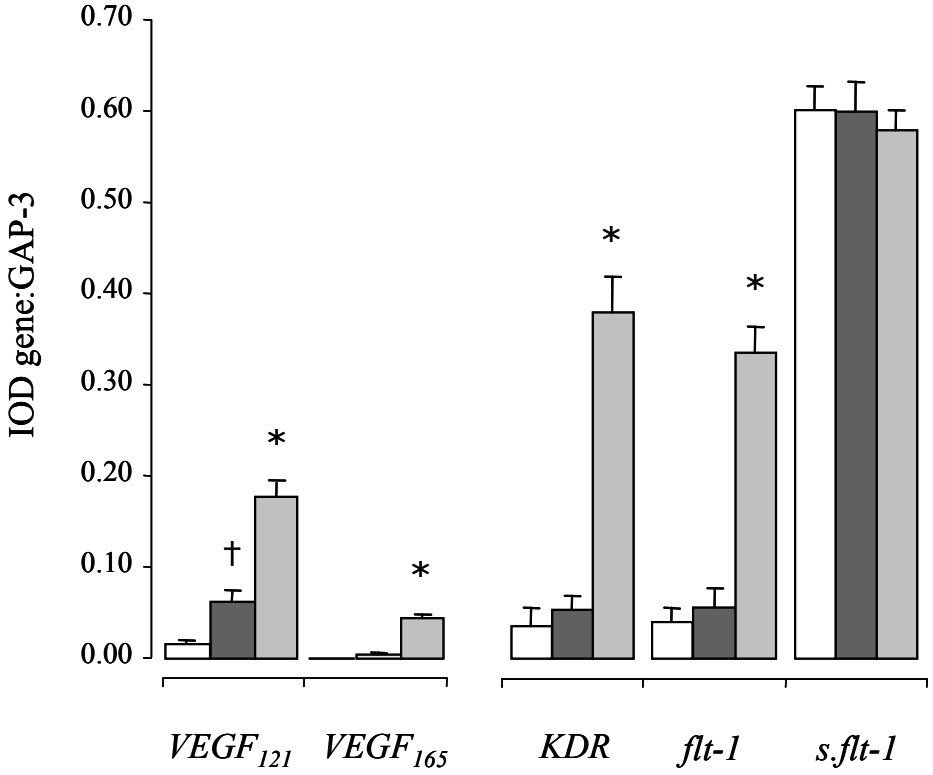


Figure 3.5 Transcription of *VEGF*-A and its receptors by SFJ status.

Control veins (open bars), VVs from individuals with a competent and functional SFJ (dark grey bars), or incompetent and dysfunctional SFJ (light grey bars). IOD gene:GAP-3 is the ratio of the integrated optical density (IOD), determined by scanning densitometry, of the specific gene product to its' corresponding product for *GAP-3*. Bars represent means values, and error-bars are s.e.m. (n=14 sections from control veins; n=29 sections analysed from VVs with a competent SFJ; n=54 sections analysed from VVs with an incompetent SFJ; all with two PCR analyses per section for each gene examined). *$p<0.001$ versus competent SFJ, and versus control vein; $^{\dagger}p<0.02$ versus control vein; all Kruskal-Wallis test.

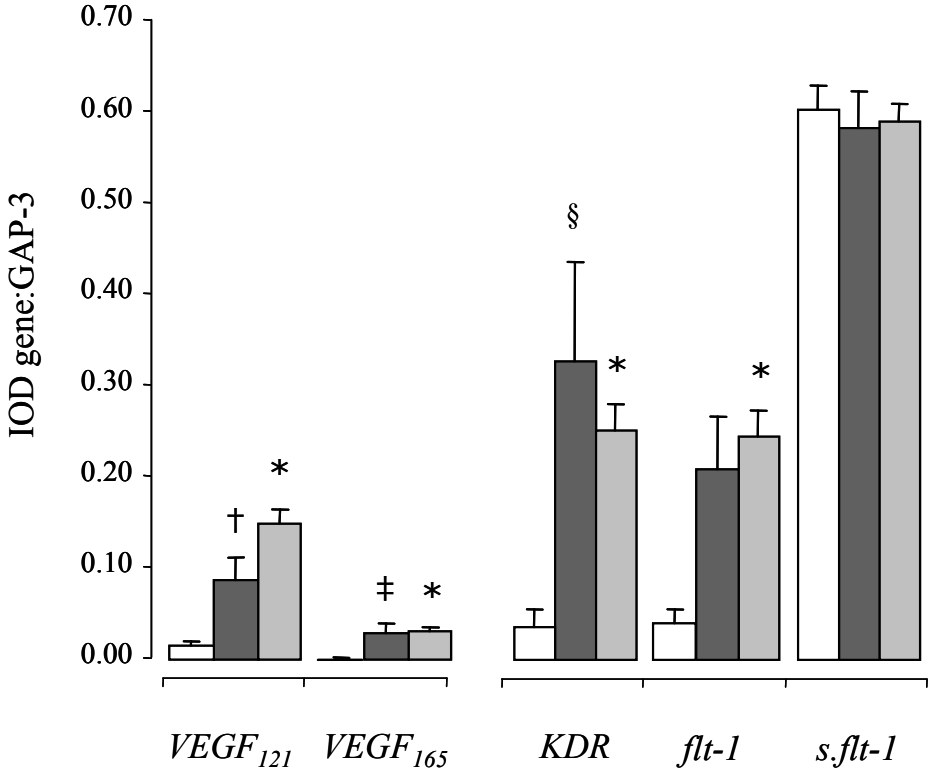


Figure 3.6 Transcription of *VEGF*-A and its receptors by segmental status.
Control veins (open bars) and VVs where the section of vein was competent and functional (dark grey bars), or incompetent and showing reflux (light grey bars). IOD gene:GAP-3 is the ratio of the integrated optical density (IOD) of the specific gene product to its' corresponding product for *GAP-3*, as determined by scanning densitometry. Bars represent means values, and error-bars are s.e.m. (n=14 sections from control veins; n=16 competent sections from VVs; n=67 incompetent sections from VVs; all with two PCR analyses per section for each gene examined). *p<0.001, $^{\dagger}p$<0.02, $^{\ddagger}p$<0.006 and $^{\S}p$<0.007 versus sections from control vein; all Kruskal-Wallis test.

Position and SFJ or segmental competence: Gene transcription was now examined according to position from where derived; as proximal, proximal-mid, mid, mid-distal and distal from the SFJ (see chapter 2.1.2. and Figure 2.1). Overall, including all sections of vein analysed, there was little or no difference in the levels of transcription of *VEGF-A*$_{121}$, *VEGF-A*$_{165}$, *VEGFR2* or *VEGFR1* dependent on position. However, there was a direct correlation for increased transcription of *s.flt-1* with descending position from the SFJ for VVs overall (p<0.04, Kendall's bivariate correlation; Figure 3.7).

To investigate further, results were subdivided by i) competence at the SFJ, and ii). segmental competence. With descending position, the competency of the SFJ had no effect on levels of transcription of *VEGF-A*$_{121}$ (Figure 3.8) *VEGF-A*$_{165}$ (Figure 3.9), *VEGFR2* (Figure 3.10) or *VEGFR1* (Figure 3.11) and no correlation was seen. However, when the SFJ was incompetent and dysfunctional, there was a correlation between descending position from the SFJ and increased transcription of *s.flt-1* (p<0.05; Figure 3.12).

When this analysis was repeated but this time examining competence of the segment, a similar pattern was observed. For competent segments, gene transcription was unaffected by position and no correlation was seen. When however, the segment was incompetent and showing reflux, there was again increased *s.flt-1* transcription correlated to descending position from the SFJ (p<0.03; Figure 3.13). In addition, there was a similar correlation in these segments of increasing transcription of *VEGF-A*$_{121}$ with descending position from the SFJ (p<0.02; Figure 3.13).

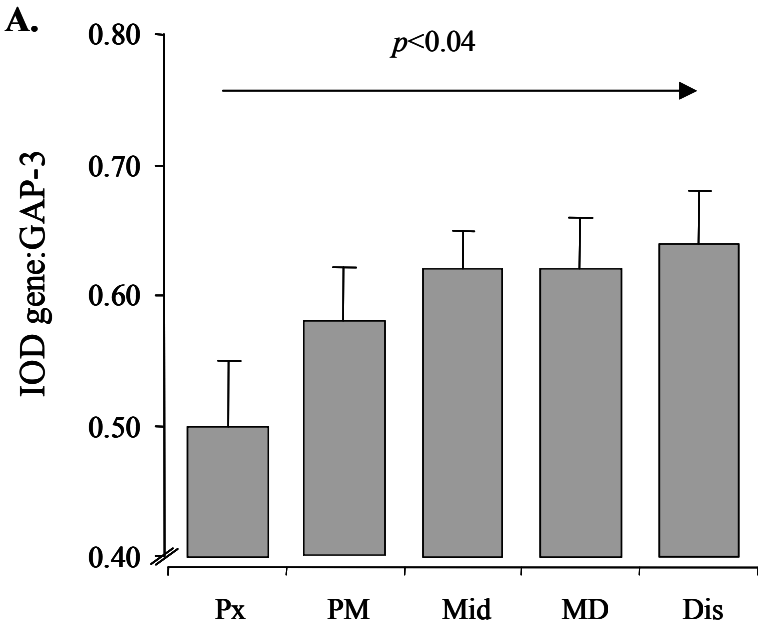


Figure 3.7 Transcription of *s.flt-1* in VVs overall by segmental position.
s.flt-1 transcription increased with descending position from the SFJ, $p<0.04$ (Kendall's bivariate correlation). IOD Gene:GAP-3 is the ratio of the integrated optical density (IOD), determined by scanning densitometry, of *s.flt-1* gene product to its' corresponding product for *GAP-3*. Bars represent means values, and error-bars are s.e.m. Position from the SFJ, (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal; all with two PCR analyses per section examined.

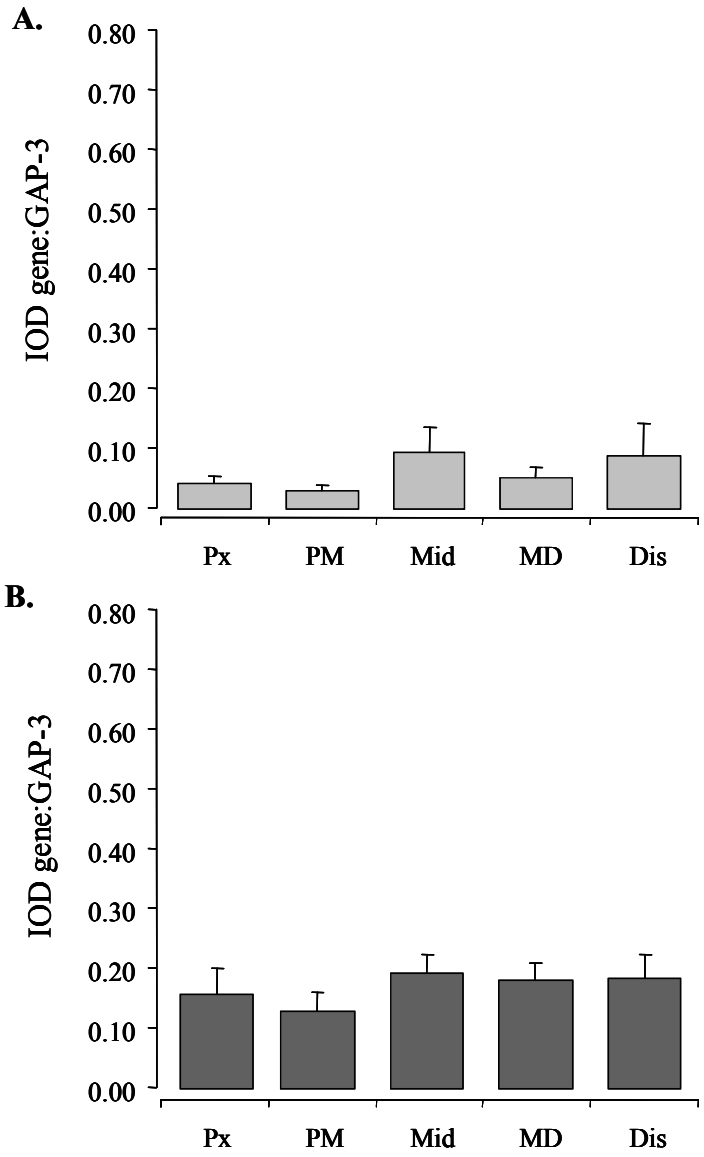


Figure 3.8 Transcription of *VEGF-A*$_{121}$ in sections of VVs by position.

Sections of VVs descending below a competent (**A**) or incompetent (**B**) SFJ. IOD gene:GAP-3 is the ratio of the integrated optical density (IOD), determined by scanning densitometry, of *VEGF-A*$_{121}$ gene product to its' corresponding product for *GAP-3*. Bars represent means values, and error-bars are s.e.m. Position from the SFJ, (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal; all with two PCR analyses per section examined.

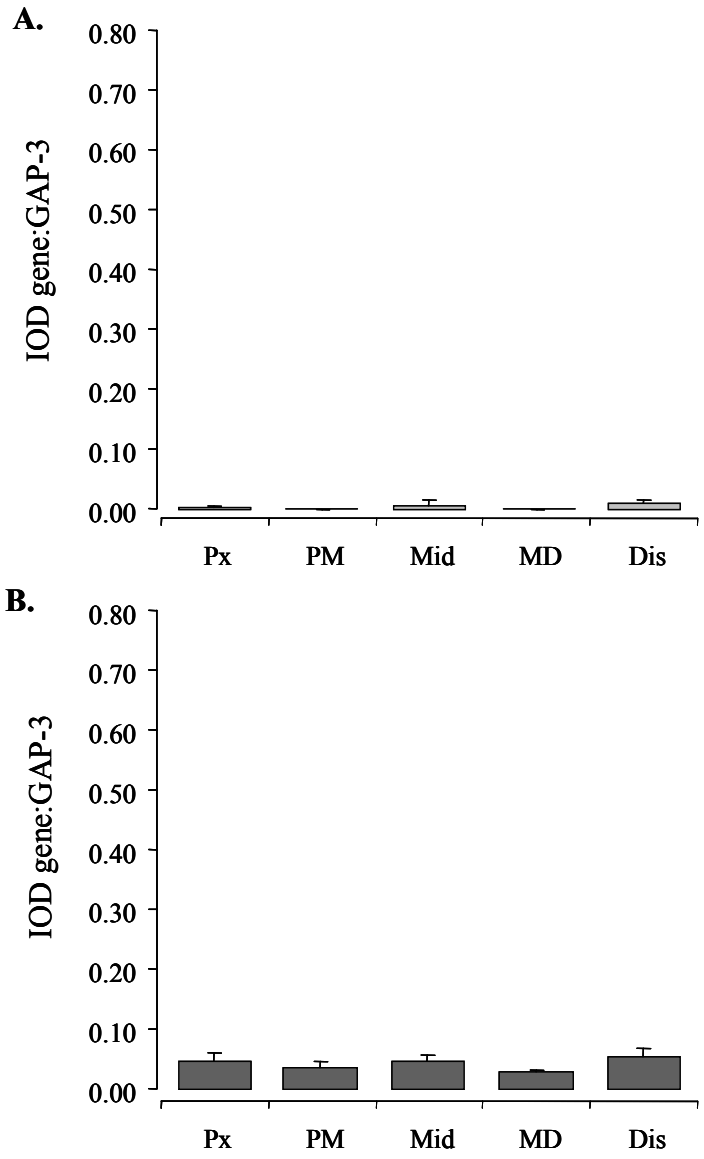


Figure 3.9 Transcription of *VEGF-A*$_{165}$ in sections of VVs by position.

Sections of VVs descending below a competent (**A**) or incompetent (**B**) SFJ. IOD gene:GAP-3 is the ratio of the integrated optical density (IOD), determined by scanning densitometry, of *VEGF-A*$_{165}$ gene product to its' corresponding product for *GAP-3*. Bars represent means values, and error-bars are s.e.m. Position from the SFJ, (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal; all with two PCR analyses per section examined.

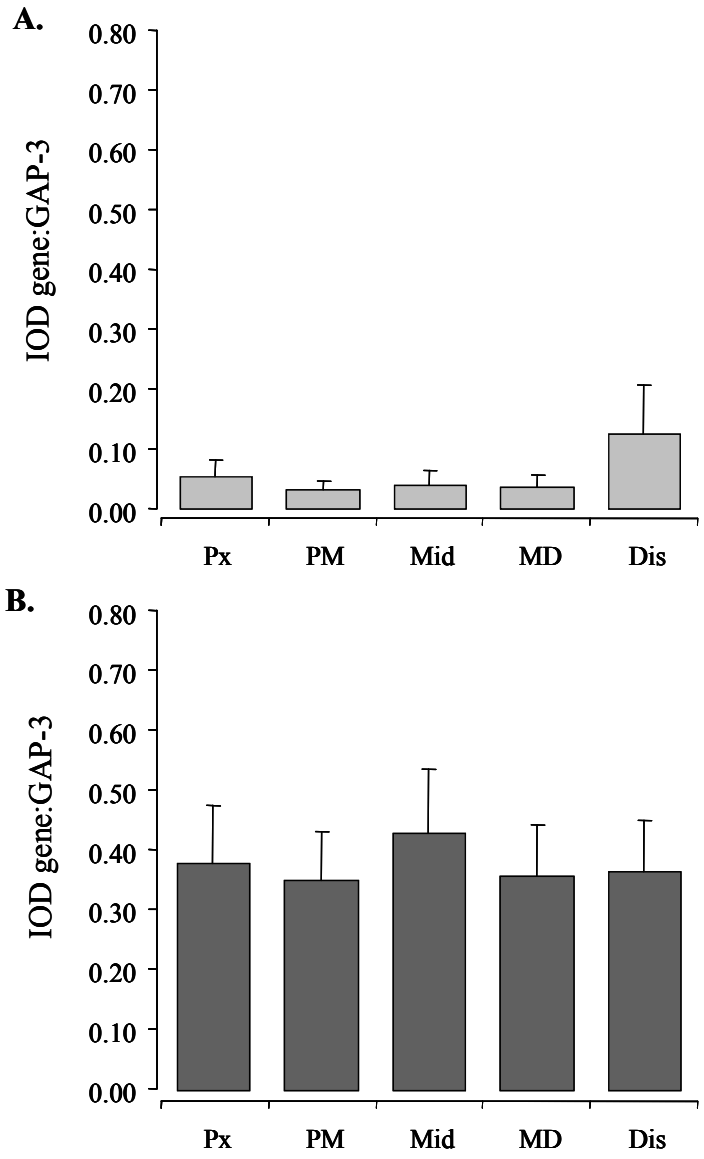


Figure 3.10 Transcription of *VEGFR2* in sections of VVs by position.

Sections of VVs descending below a competent (**A**) or incompetent (**B**) SFJ. IOD gene:GAP-3 is the ratio of the integrated optical density (IOD), determined by scanning densitometry, of *VEGFR2* gene product to its' corresponding product for *GAP-3*. Bars represent means values, and error-bars are s.e.m. Position from the SFJ, (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal; all with two PCR analyses per section examined.

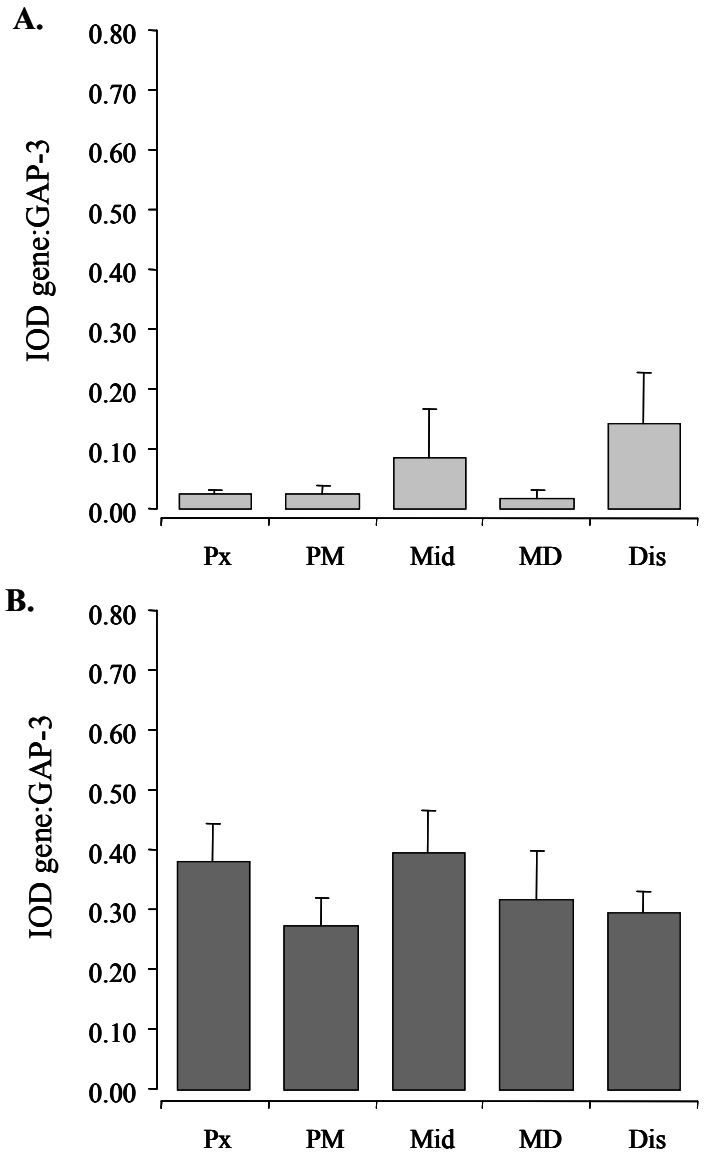


Figure 3.11 Transcription of *VEGFR1* in sections of VVs by position.

Sections of VVs descending below a competent (A) or incompetent (B) SFJ. IOD gene:GAP-3 is the ratio of the integrated optical density (IOD), determined by scanning densitometry, of *VEGFR1* gene product to its' corresponding product for *GAP-3*. Bars represent means values, and error-bars are s.e.m. Position from the SFJ, (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal; all with two PCR analyses per section examined.

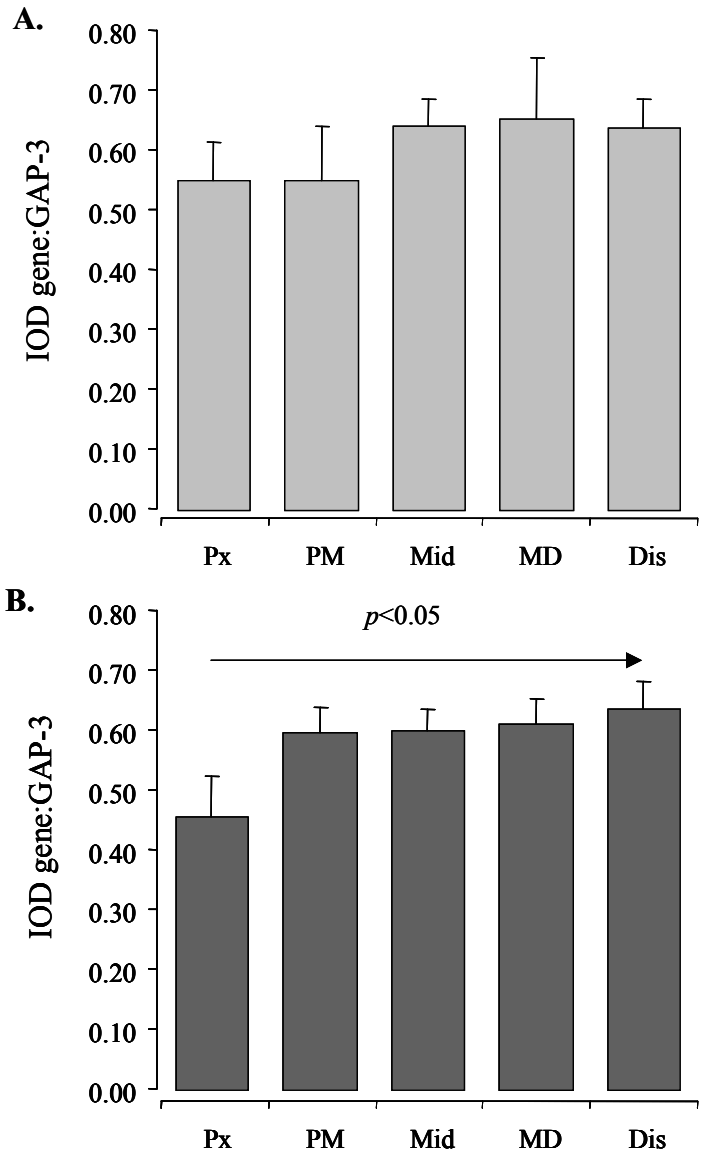


Figure 3.12 Transcription of *s.flt-1* in sections of VVs by position.

Sections of VVs descending below a competent (A) or incompetent (B) SFJ. Bars represent means values, and error-bars are s.e.m. Position from the SFJ, (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal; all with two PCR analyses per section examined. *s.flt-1* transcription increased with descending position from the SFJ, $p<0.05$ (Kendall's bivariate correlation).

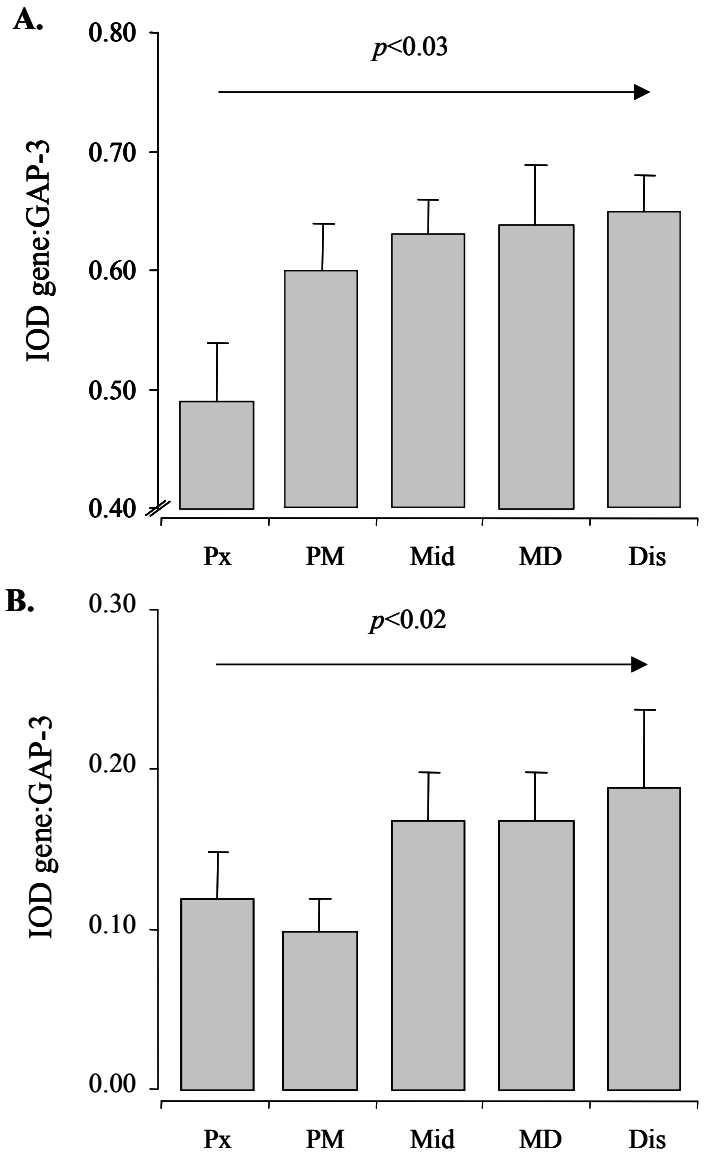


Figure 3.13 *s.flt-1* (**A**) and *VEGF-A*$_{121}$ (**B**) transcription in incompetent sections. Transcription of *s.flt-1* (p<0.03) and *VEGF-A*$_{121}$ (p<0.02) increased with descending position from the SFJ (both, Kendall's bivariate correlation). Bars represent means values, and error-bars are s.e.m. Position from the SFJ, (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal; all with two PCR analyses per section examined.

3.1.5 **Discussion**

Primary VVs are a chronic disease (Allan *et al.,* 2000), the prevalence of which increases with age (Evans *et al.,* 1998; Ruckley *et al.,* 2002), is higher in females (Evans *et al.,* 1999) and associated with a number of risk factors (see chapter 1.1.2). Incompetence of the GSV is the most common manifestation of VVs (Labropoulos *et al.,* 1999; Ruckley *et al.,* 2002) and is characterised functionally by disturbed blood flow (reflux/stasis), venous hypertension and abnormal vessel dilation (Browse *et al.*, 1999; Labropoulos *et al.,* 1999; Badier-Commander *et al.,* 2000; Ruckley *et al.,* 2002). Their pattern of appearance might suggest a progressive disease (Jones *et al.,* 1999; Brunner *et al.,* 2000) with the underlying process(es) potentially overcoming homeostatic repair mechanisms over time (Rose 1986; Venturi *et al.,* 1996) resulting in the dilated, elongated or tortuous vein observed with varicosity (Travers *et al.,* 1996; Woodside *et al.,* 2003). At the cellular level, VVs are associated with a number of structural abnormalities (see chapter 1.1.6) considered to be the result of aberrant vessel wall remodelling (Gandhi *et al.,* 1993; Venturi *et al.,* 1996; Badier-Commader *et al.,* 2000), with the processors involved influenced by the same growth factors implicated in the regulation of normal vascular homeostasis (Gale *et al.,* 1999; Yancopoulos *et al.,* 2000; Badier-Commader *et al.,* 2001). As the control of transcription of genes involved in maintaining vascular reactivity is fundamental in the regulation of homeostasis, examination of their transcriptional activity may help to elucidate the mechanism(s) underlying varicogenesis. This study is the first to examine changes in the pattern of gene transcription (VEGF-A subtypes and its receptors) in segments of VVs, matched anatomically in descending position from the SFJ, in relation to SFJ status and the underlying venous incompetence.

For VVs overall, transcription of *VEGF-A*$_{121/165}$, *VEGFR1* and *VEGFR2* were elevated compared to control veins (Figure 3.4) suggesting a potential for greater plasma VEGF-A and EC VEGF receptor expression. While transcription was elevated here, previously plasma VEGF-A was not increased in VVs (Hollingsworth *et al.,* 2001A, Howlader *et al.,* 2004), although raised plasma VEGF-A was reported in conjunction with skin changes in CVD (Shoab *et al.,* 1998; Howlader *et al.,* 2004). Protein levels were not measured here however, due in part to the difficulty in determining levels of secreted VEGF-A isoforms in the vessel wall, and as this study was undertaken specifically to investigate possible alterations in transcriptional activity of *VEGF-A* and its receptors produced by the varicose vessel wall.

In VVs, reduced contractile responses to endothelin 1 (ET-1) *in vitro* were associated with decreased ET-1 receptors, an effect regulated at the level of transcription (Barber *et al.,* 1997). The generation of VEGF-A mRNA is regulated at the level of transcription by binding of transcription factors to the *VEGF-A* gene promoter (Tischer *et al.,* 1989; Houck *et al.,* 1992; Pages *et al.,* 2005) and at the post transcriptional level by mechanisms that increase VEGF-A mRNA half life (Levy *et al.,* 1996; Levy *et al.,* 1998; see chapter 1.2.4). Consistently, an increase in VEGF-A expression in EC models affects VEGF receptor transcription, suggesting that VEGF-A may modulate the expression of its own receptors (Shen *et al.,* 1998; Wang *et al.,* 2000). Enhanced *VEGF-A* transcription but (potentially) without increased protein production (Hollingsworth *et al.,* 2001A) might suggest a problem with translation to protein. Alternatively, if the elevated levels of message (mRNA) detected here were not of full-length, the resultant protein (if produced) might potentially be truncated and so unable to bind the antibodies used for detection (Hollingsworth *et al.,* 2001A).

Further studies should address, therefore the rates of translation of VEGF-A and its receptors to protein, and the production of full-length mRNA transcripts.

The presence of VVs with levels of transcription similar to control veins (see Figures 3.1, 3.2 and 3.5), *i.e.* those VVs were the SFJ was competent and functional, provides several explanations. Firstly, aberrations in *VEGF-A/receptor* transcription may simply not be a causative mechanism in VVs development. In contrast however, a fault in the control of *VEGF-A/receptor* transcription might be involved (see chapter 1.2.2). For example, *in vitro* EC pre-incubated with VEGF-A_{165} down regulate cell surface expression of VEGFR1 and 2, which initiates elevated VEGF receptor transcription (Wang *et al.,* 2000). Notably, receptor down-regulation desensitised cells to VEGF-A_{165} activation of intracellular cell signalling pathways, associated with EC proliferation and survival (Wang *et al.,* 2000). Removal of exogenous VEGF-A_{165} allowed rapid recovery of both cell surface VEGF receptor expression as well as cellular responsiveness to VEGF-A_{165} (Wang *et al.,* 2000). In VVs, continued stimulation (from enhanced VEGF expression) in response to hypoxia, elevated pressure or disturbed blood flow may similarly induce VEGF receptor down-regulation (or induced redundancy). Furthermore, this could result ultimately in a compromised ability of the vessel wall to respond appropriately, as with ET-1 receptors in VVs *in vitro* (Barber *et al.,* 1997), an effect which may have been initiated due to elevated ET-1 production/exposure (Barber *et al.,* 1997; Mangiafico *et al.,* 1997).

A possible further explanation could be that *VEGF-A/receptor* transcription is proportional to intra-luminal pressure, so with SFJ incompetence pressure increases

and transcription becomes proportionally elevated. For this however, intra-luminal pressure in VVs with an incompetent SFJ should be greater than in those where the SFJ is functional, for which currently there is no reported evidence and should be considered for future investigations. Although unclear, the altered pattern of VEGF-A/receptor transcription seen suggests that incompetence of the SFJ may represent disease of a differing stage of development (*i.e.* later than when the SFJ is functional).

Besides intra luminal pressure, functional characteristics like disturbed blood flow (stasis or reflux) are associated with VVs and suggested to play a role in vessel wall remodelling (Browse *et al.,* 1999; Badier-Commader *et al.,* 2001; Wali *et al.,* 2002B). Comparisons of patterns of incompetence with the distribution of varicosities using colour flow duplex ultrasound examination suggests that the development of VVs may occur by a process of 'spreading incompetence' (Cooper *et al.,* 2003) potentially originating from an initial focal point(s) (Labropoulos *et al.,* 1999). This is substantiated by observations that the presentation of varicosity is often patchy and irregular (Badier-Commader *et al.,* 2001), with varicose segments of vessel wall interspersed with apparently normal segments (see chapter 1.1.6). To further investigate this, the pattern of gene transcription was examined in relation to the competency (reflux or no reflux) of the vessel wall segment along the vein. When examining segmental competence (in contrast to SFJ competency), the pattern of transcription seen was different. Transcription of *VEGF-*$A_{121/165}$, *VEGFR2* and *VEGFR1* was similar in both competent and incompetent segments of VVs, but was elevated in all segments (competent or incompetent) compared to control (Figure 3.6). Activation of VEGF-A/receptor transcription as a homeostatic response might

therefore, reflect an earlier event occurring prior to the development of incompetence within the vein wall.

VEGF-A, produced from the vSMC in response to hypoxia, disturbed blood flow or pressure, stimulates production of NO from the vascular endothelium (Dulak *et al.,* 2003; Kimura *et al.,* 2003). NO, itself released from the endothelium due to mechanical stimuli like shear stress associated with disturbed flow (Luscher 1991; Bundy *et al.,* 2000) and hypoxia (Pearson *et al.,* 1998; Kimura *et al.*, 2000), in turn acts on vSMC to produce VEGF-A, generating a 'loop' mechanism to mediate vascular reactivity (Servos *et al.,* 1999, Bundy *et al.,* 2000; Kimura *et al.,* 2003). Again, as with ET-1, (Barber *et al.,* 1997; Mangiafico *et al.,* 1997) enhanced *VEGF-A/receptor* transcription in response to 'stimulus' like hypoxia, disturbed blood flow or pressure, if not relieved or controlled appropriately, might lead ultimately to altered VEGF-A/receptor activity and vein wall to responses. Furthermore, continued and elevated VEGF-A/receptor expression (via the mechanism described above) may lead to elevated production of nitric oxide (Servos *et al.,* 1999) resulting in the generation of other reactive oxygen species, and so contributing to the further development of pathology. Therefore, in trying to maintain homeostasis, the activation of *VEGF-A/receptor* transcription may itself become a central part of the subsequent pathological process.

A surprising result was that transcription of *s.flt-1* was similar in all samples/segments and unaffected by varicosity or competence (Figure 3.5 and 3.6). The importance of control of VEGF-A in the pathogenesis of vascular disorders has been realised through isolation of a soluble truncated extra-cellular isoform of

VEGFR1, s.flt-1 (Kendall *et al.,* 1993; Kondo *et al.,* 1998). Soluble flt-1 modulates the action of VEGF by sequestering free VEGF-A and so preventing binding to trans-membrane receptors (Kendall *et al.,* 1993; Kendall *et al.,* 1996A; Siemester *et al.,* 1998; Barleon *et al.,* 2001) and by reducing the activation status (phosphorylation) of VEGFR2 (Graubert *et al.,* 2001), possibly by forming stable inactive heterodimers (Kendall *et al.,* 1996A; see chapter 1.2.3). Hypoxia can both stimulate the production of VEGF-A (Shima *et al.,* 1995; Levy *et al.,* 1996) and up-regulate s.flt-1 protein production (Liu *et al.,* 1995; Gerber *et al.,* 1997), whilst down-regulating auto-phosphorylation (hence, activation) of VEGFR1 (Ahmed *et al.,* 2000; Graubert *et al.,* 2001). Potentially therefore, hypoxia may stimulate in conjunction with the production/release of VEGF-A, an associated down-regulation of VEGFR1 in favour of s.flt-1 to control the action of the VEGF-A produced (Kendall *et al.,* 1993; Kondo *et al.,* 1998).

Transcription of *s.flt-1* increased in VVs overall with descending position from the SFJ (Figure 3.7), and with SFJ incompetence (Figure 3.12). Similarly, this pattern of increased *s.flt-1* transcription was observed descending from the SFJ in incompetent segments (Figure 3.13A). Therefore, if intra-luminal pressure increases with descending position from the SFJ, enhanced *VEGF-A* transcription might be expected and accordingly, enhanced *s.flt-1* transcription to mediate VEGF-A action (Kendall *et al.,* 1993; Kondo *et al.,* 1998). However, elevated *VEGF-A* transcription was not observed with descending position from the SFJ (Figure 3.8 and 3.9), although this may be masked by the overall elevated levels of *VEGF-A* transcription seen in VVs (Figure 3.4). Alternatively, enhanced *s.flt-1* transcription may simply reflect an on-going pathological process. Previously, we have observed that varicosities tend to

appear more distally, and at a position at, or below, the underlying venous incompetence (Labropoulos *et al.,* 1999; Cooper *et al.,* 2003). Collectively, the pattern of disease, in conjunction with alterations in gene transcription, might reflect a process of increasing pressure from proximal to distal, but with disease and the appearance of varicosities developing in a distal to proximal manner. Descending position from the SFJ correlated in incompetent segments with increased transcription also, of *VEGF-A*$_{121}$ (Figure 3.13B); notably the only gene to be elevated transcriptionally in VVs with a competent SFJ (Figure 3.5). The function of VEGF-A$_{121}$ is currently unclear however, although structurally it is less effective at inducing EC proliferation than VEGF-A$_{165}$ (see chapter 1.2.3) enhanced transcription has been observed associated with inflammatory disease.

Although the results reported here, may suggest a potential problem with VEGF responses in VVs they should be interpreted with caution. Firstly, the results are based on small patient numbers and it would be more informative to extend these studies to more individuals, specifically those with an "earlier" stage of varicose disease *i.e.* VVs with competent SFJ and/or vessel wall segments. In addition, the use of control samples from individuals undergoing surgery for cardiovascular disease, while not the most appropriate were the most suitable control available. Although great care was taken when correlating harvested GSV to duplex scan reports, the inherent variability of the diseased vessel together with the difficulties in marking sites of reflux, suggests that certain alignments may not be completely accurate. Finally, investigation of the possible mechanism(s) of transcriptional induction (transcriptional activators) would help clarify whether *VEGF-A/receptor* transcription is indeed causative or merely a reflection on the on-going disease process(es).

Summary points

The results of this study can be summarised as follows:

1. The transcription of all genes examined except *s.flt-1* were elevated in sections of VVs compared to those from control veins.

2. Transcription of the secreted VEGF-A isoforms (*VEGF-A*$_{121}$ and *VEGF-A*$_{165}$) and receptors (*VEGFR2* and *VEGFR1*) were elevated in VVs when the SFJ was incompetent.

3. Transcription was similarly elevated in VVs irrespective of segmental competence.

4. There was no change in transcription of *s.flt-1* with SFJ or segmental incompetence although transcription increased with descending position from SFJ.

5. Altered transcription of *VEGF-A* and its receptors (notably *VEGF-A*$_{121}$ and *s.flt-1*) maybe associated in some part, with the process(es) of varicogenesis and represent an earlier event, before the development of vessel wall incompetence.

6. In contrast, SFJ incompetence may represent a later stage of disease development, or a differing aetiology.

3.2 **RELEASE OF S.FLT-1 WITH AN INDUCED STASIS**

3.2.1 **Background**

Although an imbalance between the mediators of vascular tone has been proposed as a causative mechanism in primary VVs (Schuller-Petrovic *et al.,* 1997; Brunner *et al.,* 2001), it is unclear where the fault lies, *i.e.* with the production of these mediators, the vessels ability to respond appropriately to them when produced or indeed an inability to switch off activating VEGF-A signals. Previously, an increase in luminal pressure (application of a cuff) resulted in a rapid elevation of plasma VEGF-A in control subjects, but not in patients with VVs (Hollingsworth *et al.,* 2001A). However, both elevated transcription (see chapter 3.1.4) and VEGF-A protein (Shoab *et al.,* 1998; Howlader *et al.,* 2004) are associated with VVs, suggesting potentially either a loss of response to increased pressure in these veins or rapid clearance of the released VEGF-A. Notably, the transcription of *s.flt-1* (soluble isoform of VEGFR1 membrane receptor) was similar elevated in VVs, with increased transcription associated with decreasing position below an incompetent SFJ (see chapter 3.1.4). VEGF-A mediates its dilatory and mitogenic effects through activation of VEGFR2 (Millauer *et al.,* 1993) the action of which potentially is modulated by s.flt-1, which binds VEGF-A with high affinity and modulates VEGF-A signalling (Kendall *et al.,* 1993; Belgore *et al.*, 2000).

3.2.2 **Aim**

To examine baseline levels of plasma s.flt-1 in the GSV and its release following a mild experimentally induced venous hypertension and whether release is affected by varicosity.

3.2.3 **Methods**

This study was conducted with approval of the Joint UCL/UCLH Committees on the Ethics of Human Research. Patients attending The Vascular Unit, The Middlesex Hospital, London for assessment for primary VVs and control subjects (selected from staff within the Department of Surgery) were asked to participate and if agreeable, written consent obtained (see Chapter 2.1.1).

A mild, venous stasis was experimentally induced as described in chapter 2.1.2. This method was used for a number of experimental reasons. Ideally, to induce venous stasis, patients and subjects would have stood upright for 20-25 minutes before blood samples were obtained. However, this was not practical as patients were reluctant to stand for prolonged periods on a voluntary basis. Furthermore, the intent experimentally was to induce a mild and localised change in the venous blood flow, rather than a more severe venous stasis as might be expected from prolonged standing. The 'cuffing' time of 10 minutes was chosen to observe how quickly changes in plasma factors might be brought about under such conditions and therefore, a closer approximation of how the vein might react to minor changes in localised blood flow. Before and following venous stasis, blood samples were collected and processed as described in chapter 2.1.2.

From plasma, levels of s.flt-1 were determined using a quantitative sandwich enzyme immunoassay kit (Quantikine™ human sVEGF R1; R&D Sytems, UK) according to the manufacturer's instructions as described in chapter 2.4.3.

Statistical analysis of results was undertaken as described in chapter 2.6.2.

3.2.4 **Results**

Patient demographics: 32 subjects were recruited, of which 13 (of 21) patients with primary VVs and 7 (of 11) control subjects yielded analysable results (Table 3.2.); samples and results were not obtained for all subjects recruited due to either difficulties in obtaining blood from the foot, or coagulation of samples due to limited volumes obtained. Samples from individuals were only included for analysis if they produced on repeated and separate assays, results with less than a 10% inter-experimental variation.

Before cuff: Baseline levels of plasma s.flt-1 in samples obtained from the arm were similar in both control subjects (median, range: 35.4, 30.1 – 41.0 pg/ml) and patients with primary VVs (33.1, 24.3 – 48.5 pg/ml; $p>0.1$). In control subjects, baseline s.flt-1 in samples obtained from the foot before application of the below-knee cuff vein (33.2, 25.0 – 37.0 pg/ml) were similar to those obtained from the arm (35.4, 30.1 – 41.0 pg/ml; $p>0.1$; Figure 3.14.). In contrast however, levels of s.flt-1 in plasma samples obtained from the foot of patients with primary VVs before cuff application (51.1, 39.7 – 89.4 pg/ml) were markedly elevated compared to those from the arm (33.1, 24.3 – 48.5 pg/ml; foot before versus arm, $p<0.001$) and baseline samples from control subjects (foot before control versus varicose, $p<0.001$; see Figure 3.14. and Figure 3.15.).

After cuff Application of the below-knee cuff for 10 minutes resulted in an overall mean (± s.e.m) increase of 54.2 (± 22.5) % in the levels of plasma s.flt-1 in control subjects from 33.2 (25.0 – 37.0) pg/ml to 44.3 (32.5 – 87.4) pg/ml (before versus after, $p<0.015$; Figure 3.14).

Description	Varicose	Control
patients, *n* =	13	7
median age (range) yrs	46* (21-69)	32 (22-55)
male, *n* =	4	5
female, *n* =	9	2

Table 3.2 Patient demographics and samples for analysis of plasma s.flt-1.
32 subjects were recruited, of which 13 (of 21) patients with primary VVs and 7 (of 11) control subjects yielded analysable results. *$p<0.1$ versus control, Mann & Whitney U test.

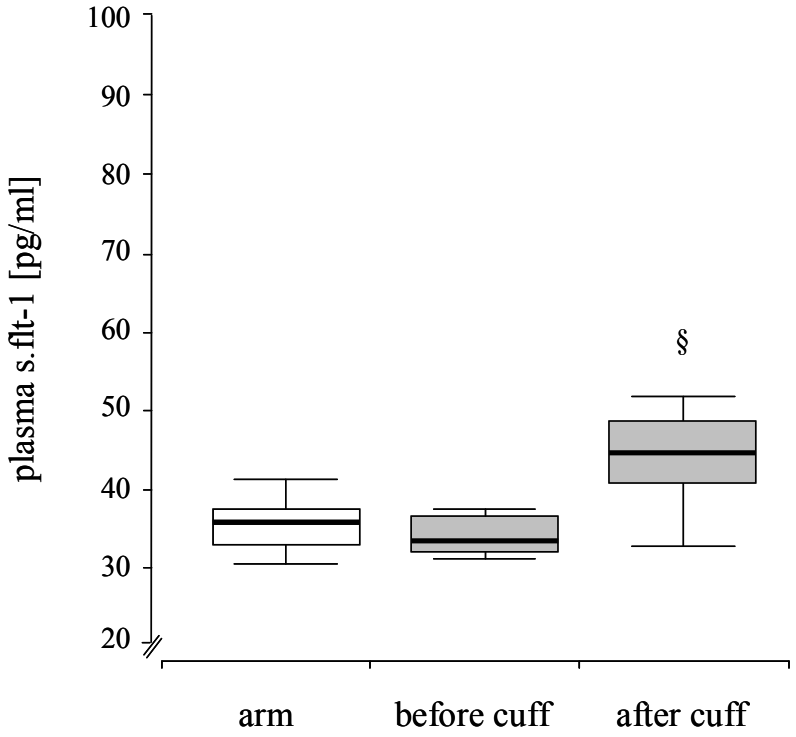


Figure 3.14 Levels of plasma s.flt-1 detected in blood of control subjects.
Blood taken from arm (open bars) or the foot veins (grey bars) before and after application of a below-knee cuff. For each subject, duplicate assays were performed, each with at least two replicates per assay, and the mean value used for further analysis. Box-plots represent thick line (median), boxes (inter-quartile range) and error bars (range), § $p<0.015$ versus control subjects 'before cuff' Kruskal-Wallis test.

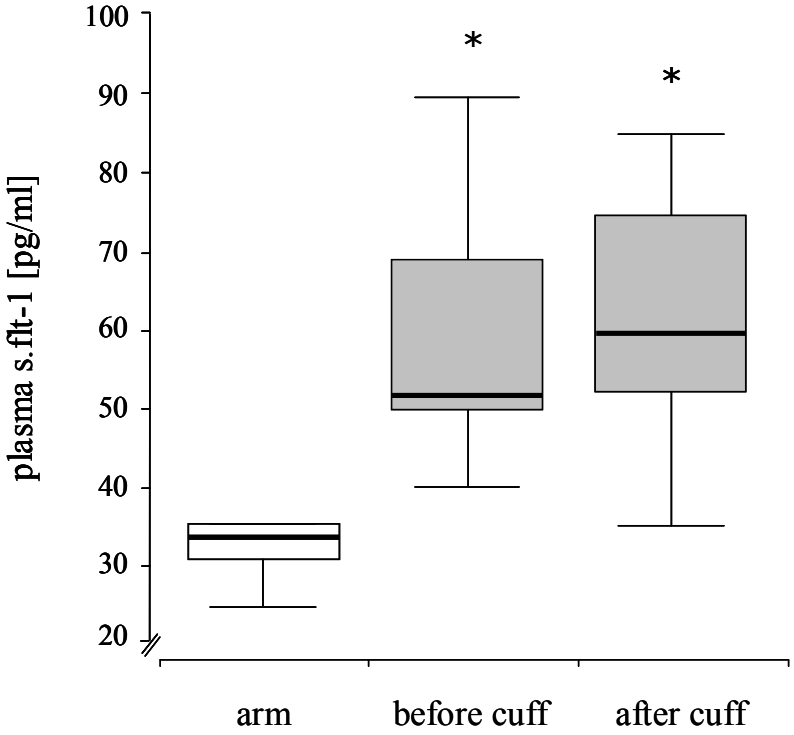


Figure 3.15 Plasma levels of s.flt-1 in blood from patients with VVs.

Bloods taken from arm (open bars) or the foot veins (grey bars) before and after application of a below-knee cuff. For each subject, duplicate assays were performed, each with at least two replicates per assay, and the mean value used for further analysis. Box-plots represent thick line (median), boxes (inter-quartile range) and error bars (range). *p<0.001 versus VVs 'before cuff', and control subjects 'before cuff' Kruskal-Wallis test.

However, in patient's with primary VVs there was little or no change in plasma s.flt-1 following application of the cuff, these being only marginally elevated; with an overall mean increase of 5.5 (± 5.8) % from 51.1 (39.6 – 89.4) pg/ml to 59.2 (34.7 – 126.4) pg/ml (see Figure 3.15).

3.2.5 **Discussion**

Application of a cuff and the resulting venous stasis should induce a change in luminal pressure and local blood chemistry in the vein, the normal homeostatic response to which should be vessel wall dilation and/or increased vessel permeability (Micheils *et al.,* 1997; Browse *et al.,* 1999). For dilation, the production of vasodilators like VEGF-A and NO would be expected (Servos *et al.,* 1999). The production of VEGF-A by the vessel wall would induce a concomitant increase in the expression of the VEGF receptors in the vascular endothelium (Wang *et al.,* 2000) including the production of s.flt-1 (Barleon *et al.,* 1997A; Chen *et al.,* 2000B) as a possible mechanism to attenuate the action of VEGF-A (Kendall *et al.,* 1993).

Soluble flt-1, the truncated extra-cellular isoform of VEGFR1 (Kendall *et al.,* 1993; Kondo *et al.,* 1998) can modulate the action of VEGF via at least two known mechanisms: i) by binding to, and thus sequestering free VEGF-A and so preventing its ligation to the trans-membrane receptors (Kendall *et al.,* 1993; Barleon *et al.,* 1997B; Ferrara *et al.,* 1998; Siemester *et al.,* 1998B; Barleon *et al.,* 2001), ii) by reducing the activation status (phosphorylation) of VEGFR2 (Siemester *et al.,* 1998B; Graubert *et al.,* 2001), possibly by forming stable inactive heterodimers (Kendall *et al.,* 1996A; see chapter 1.2.3). Evidence for the modulation of VEGF-A by s.flt-1 was observed during endometrial regeneration during menstruation (Clarke *et al.,*

1998) where complexes of s.flt-1 bound to VEGF are detected in plasma during the early menstrual phase of the cycle (Graubert *et al.,* 2001). Furthermore, with progression to the late menstrual and early proliferative phase, a reduction in plasma s.flt-1 was associated with elevated levels of plasma VEGF-A (Graubert *et al.,* 2001). Similarly, in patients with peripheral arterial disease, a significant reduction in baseline levels of plasma s.flt-1 was associated with elevated plasma VEGF-A (Belgore *et al.,* 2001A; Blann *et al.,* 2002). Furthermore, in hypertensive patients elevated VEGF-A with reduced s.flt-1 was associated with increased endothelial damage and cardiovascular risk (Felmeden *et al.,* 2003); the decrease of which following intensive management was associated with an increase in plasma s.flt-1 and a concomitant lowering of plasma VEGF-A levels (Felmeden *et al.,* 2003).

In contrast, elevated VEGF-A was not associated with a reduction in plasma s.flt-1 in patients with coronary arterial disease (Blann *et al.,* 2002) and plasma VEGF-A and s.flt-1 were both elevated in patients with (uncomplicated) essential hypertension, and both decreased following treatment (Belgore *et al.,* 2001A). Although levels of plasma VEGF-A and s.flt-1 increase with the progression of normal pregnancy (McKeenan *et al.,* 2004), a marked elevation in s.flt-1 occurs earlier and in the absence of any change in circulating VEGF-A, during early gestation in preeclampsia (McKeenan *et al.,* 2004). However, in patients with preeclampsia, systemic plasma s.flt-1 is further increased with progression of pregnancy (Maynard *et al.,* 2003) and elevated levels of sflt-1 are associated with a marked reduction in circulating free VEGF (Maynard *et al.,* 2003). Interestingly, during vascular repair after menstruation, the activation of VEGFR2 (phosphorylation) was inversely correlated

with the presence of s.flt-1 suggesting a more direct role in attenuating VEGFR2 mediated signalling by s.flt-1 (Graubert *et al.,* 2001).

In control subjects, as for VEGF-A previously (Hollingsworth *et al.,* 2001A), a mild venous stasis induced an increase in the levels of plasma s.flt-1 within 10 minutes (see Figure 3.14.). VEGFR1 and s.flt-1, like VEGF-A contain a hypoxia response element in their promoter region and are potently up-regulated by hypoxia (Liu *et al.,* 1995; Gerber *et al.,* 1997). Hypoxia also down-regulates the activation (phosphorylation) of membrane bound VEGFR1 which is thought to elevate the production of s.flt-1 (Ahmed *et al.,* 2000). However, s.flt-1 is produced *de novo* as an alternative splice variant (Kendall *et al.,* 1993; Kondo *et al.*, 1998) and even though post transcriptional modulation of polyadenylation sites by the spliceosome is suggested to elevate s.flt-1 mRNA expression (Huckle *et al.,* 2004), the rapid increase in s.flt-1 protein seen her after 10 minutes is unlikely to have been produced by this route.

Alternatively, the increased level of s.flt1 protein detected in control subjects might have been derived by release from bound VEGF:s.flt-1 complexes present within the plasma (Belgore *et al.,* 2001B). The dissociation of these complexes under increased oxygen tension could liberate both VEGF and s.flt-1, and help to explain the elevation of VEGF and s.flt-1 in the plasma of patients with VVs. However, the stochiometry does not support this as a mechanism, as levels of s.flt-1 (picograms) are an order of magnitude less than that detected for VEGF-A (nanograms) in VVs. So, as for hypoxia induced transcriptional activation, s.flt-1 release from bound VEGF:s.flt-1 complexes are unlikely to account for our results here after 10 minutes

and further examination is required to interpret the results obtained. To clarify the possible mechanism(s) underlying the rapid elevation in s.flt-1 protein observed in VVs, further investigations including analysis post-translational modifications and protease activity would be of benefit.

In patients with primary VVs, the baseline level of s.flt-1 were markedly elevated in the foot vein before application of the cuff compared to those from the arm ($p<0.001$) and baseline levels in control subjects ($p<0.001$, see Figure 3.14.). This suggests that elevated s.flt-1 probably reflects a more localised situation associated with the site of disease in VVs instead of a systemic problem (Goldman *et al.*, 1998; Belgore *et al.,* 2001B; Hoar *et al.,* 2004). We did not observe elevated levels of VEGF in VVs (Hollingsworth *et al.,* 2001A), but elevated VEGF-A was reported in peripheral venous disease, primarily in the presence of skin changes (Shoab *et al.,* 1998; Howlader *et al.,* 2004) and with chronic critical limb ischaemia (Choksy *et al.,* 2004). Although plasma s.flt-1 was not determined in these studies, another potential explanation for elevated s.flt-1 in VVs could be simply a reflection of an ongoing inflammatory process in the vein. If so the elevated s.flt-1 observed in patients with VVs would be due to factors including pro-inflammatory cytokines, alterations to pH or reactive oxygen species localised more to the site of disease.

With application of the below-knee cuff to patients with VVs, a slight non-significant increase in s.flt-1 was observed, similar to that observed previously for VEGF-A in these subjects (Hollingsworth *et al.,* 2001A). A lack of increase in s.flt-1 might reflect a position whereby as the baseline levels are already significantly elevated, there is a reduced ability to elevate these further; for example, responses are already

near maximal, or that control of the mechanism enhancing s.flt-1 production is compromised.

The results reported here, extend the earlier observations suggesting a potential problem with VEGF responses in VVs. There are however, several caveats to this study. Firstly, the results are based on small samples numbers and it would be more informative to extend these studies to more individuals. Further, investigation of the relationships between VEGF-A and s.flt-1 with reference to C.E.A.P. grading would help to clarify if the effects seen are causative rather than simply a reflection of on-going disease processes. Finally, a more detailed examination of s.flt-1 mRNA half-life, rates of protein translation, and potential post-translation modifications would aid our understanding of the role of s.flt-1 and its' control of VEGF in the development and progression of VVs.

Summary points

The results of the studies performed here can be summarised as follows:

1. Plasma levels of s.flt-1 are rapidly increased in control subjects following an induced stasis, but not in patients with primary VVs.

2. Baseline levels of plasma s.flt-1 are elevated in patients with primary VVs before application of a cuff.

3.3 ***B-CATENIN*** **TRANSCRIPTION AND PROTEIN EXPRESSION**

3.3.1 **Background**

Normal VEGF-A signalling (via VEGFR2) dismantles the endothelial adherens junction (Esser *et* al 1998; Kevil *et al.,* 1998) resulting in a loss of cadherin mediated cell-cell adhesion (Cohen *et al.,* 1999) and increased endothelial permeability (Kevil *et al.,* 1998) with the release β-catenin into the cytoplasm (Moon *et al.,* 2004; Nelson *et al.,* 2004; see chapter 1.3.2.). In the absence of a stabilisation signal, cytoplasmic β-catenin is rapidly degraded (Aberle *et al.*, 1997; Moon *et al.,* 2004) and as such is rarely detected in the adult vasculature (Goodwin *et al.,* 2002). However in the presence of a stabilisation signal, β-catenin translocates to the nucleus and acts as a potent transcriptional activator of various genes implicated with cellular proliferation including c-myc and cyclin D1 (He *et al.,* 1998; Beherens *et al.,* 1996; see chapter 1.3.1.). The cadherin/catenin complex therefore, is suggested to regulate the activity of β-catenin signalling by retaining bound β-catenin in the AJ and preventing its translocation to the nucleus for targeted gene expression (Gottardi *et al.,* 2004; Dejana *et al.,* 2004). If as suggested, VEGF-A signalling is disturbed with the initiation of VVs, alterations in the normal functioning of the protein in the regulation of vascular homeostasis should be observed in VVs, *i.e.* EC proliferation survival and permeability (see chapter 1.2.6.2). Dysfunction in VEGF-A signalling may therefore predispose to elevated β-catenin, and so the potential for altered gene expression associated with vascular remodelling in primary VVs.

3.3.2 **Aim**

To examine the pattern of activity of β-catenin in primary VVs, specifically in relation to the underlying venous incompetence using samples previously assessed for VEGF-A and the VEGF receptors.

3.3.3 **Methods**

This study was conducted with approval of the Joint UCL/UCLH Committees on the Ethics of Human Research as described in chapter 2.1.1. Samples of varicose GSV were obtained from patients undergoing sapheno-femoral ligation and stripping of the long saphenous vein for the treatment of primary VVs. Control GSV was obtained from patients undergoing cardiac bypass, and who had no clinical evident symptoms of varicose disease in either limb (see chapter 2.1.2).

Samples of varicose or control GSV collected on ice at operation were processed as described in chapter 2.1.2. Total RNA was extracted with TRI Reagent™ (see chapter 2.2.2) and then reverse transcribed to cDNA as described in chapter 2.3.1. Total protein was extracted as described in chapter 2.2.3 and separated by reducing and denaturing SDS-PAGE (see chapter 2.4.5).

Transcription of *β-catenin*, *c-myc* and *cyclin D1* was examined by semi-quantitative PCR (see chapter 2.3.4 and representative gel pictures Figures 3.16-18) and analysed by scanning densitometry as described in chapter 2.5.2. The expression of β-catenin protein was examined by semi-quantitative Western Blot (see chapter 2.4.7 & 2.4.8 and representative western blots Figures 3.16-18) and analysed by scanning densitometry as described in chapter 2.5.3.

Statistical analysis of results was undertaken as described in chapter 2.6.3.

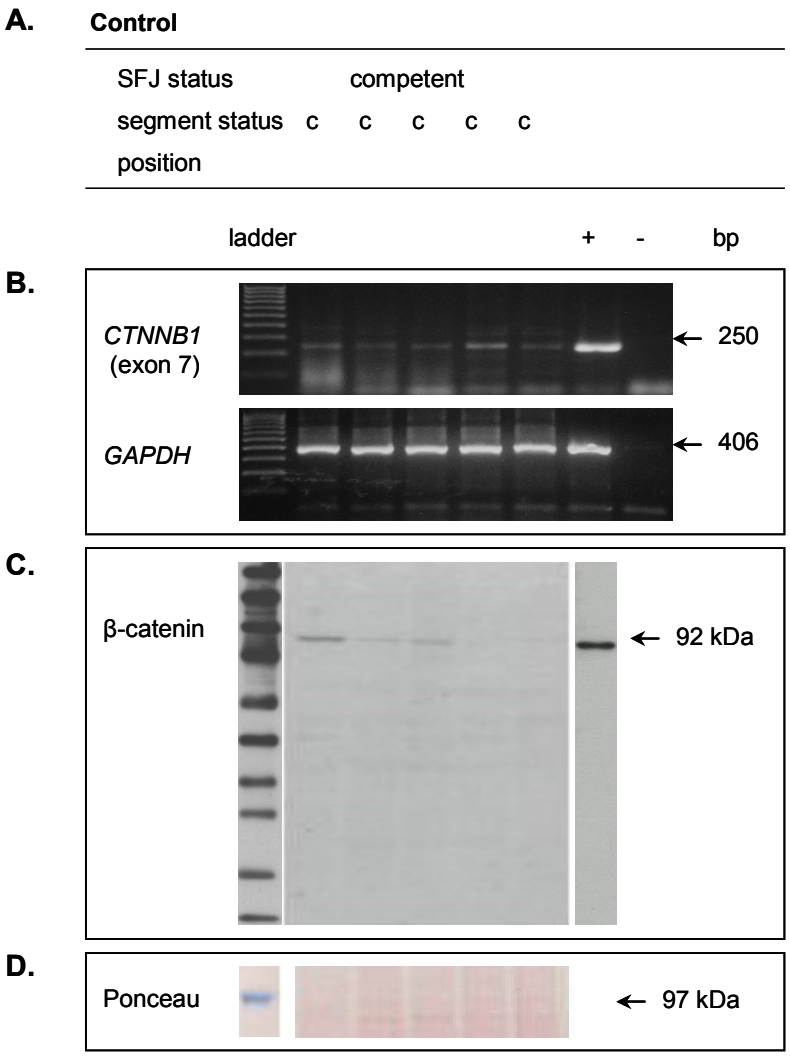


Figure 3.16 β-catenin gel and western blot of VVs sections in control vein segments. For each individual segment analysed, the competency of the SFJ and vessel wall was determined from duplex scan and classified as being competent with no reflux (c) or incompetent with reflux evident (i) (**A**). *β-catenin* gene (*CTNNB1*) transcription was examined by semi-quantitative RT-PCR. Products were visualised by agarose electrophoresis and the IOD of each band measured by scanning densitometry. Gene transcription was reported as IOD ratio of *β-catenin* PCR product normalised against control house keeping gene *GAP-3* (*GAPDH*) (**B**). β-catenin protein was detected with antibody and determined by western blot (**C**). β-catenin protein was measured by scanning densitometry and reported as IOD ratio of β-catenin protein normalised to total protein loaded (Ponceau S) (**D**).

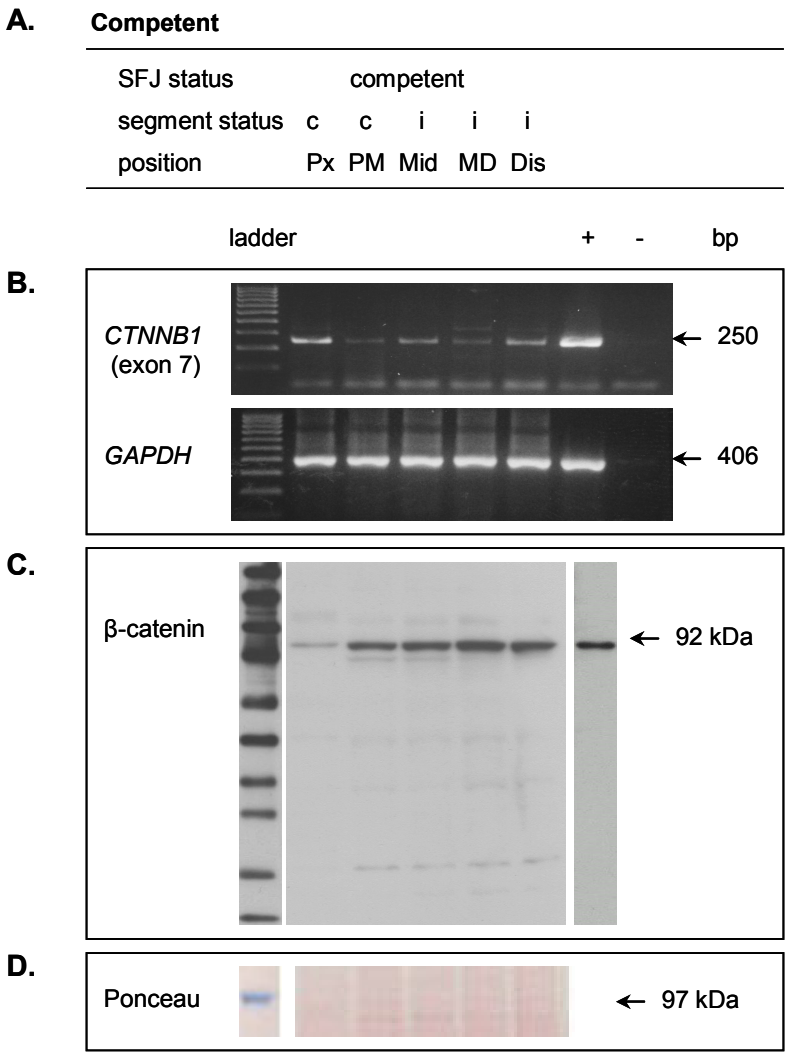


Figure 3.17 β-catenin gel and western blot of VVs sections with a competent SFJ. For each individual segment analysed, the competency of the SFJ and vessel wall was determined from duplex scan and classified as being competent with no reflux (c) or incompetent with reflux evident (i) (**A**). *β-catenin* gene (*CTNNB1*) transcription was examined by semi-quantitative RT-PCR. Products were visualised by agarose electrophoresis and the IOD of each band measured by scanning densitometry. Gene transcription was reported as IOD ratio of *β-catenin* PCR product normalised against control house keeping gene *GAP-3* (*GAPDH*) (**B**). β-catenin protein was detected with antibody and determined by western blot (**C**). β-catenin protein was measured by scanning densitometry and reported as IOD ratio of β-catenin protein normalised to total protein loaded (Ponceau S) (**D**).

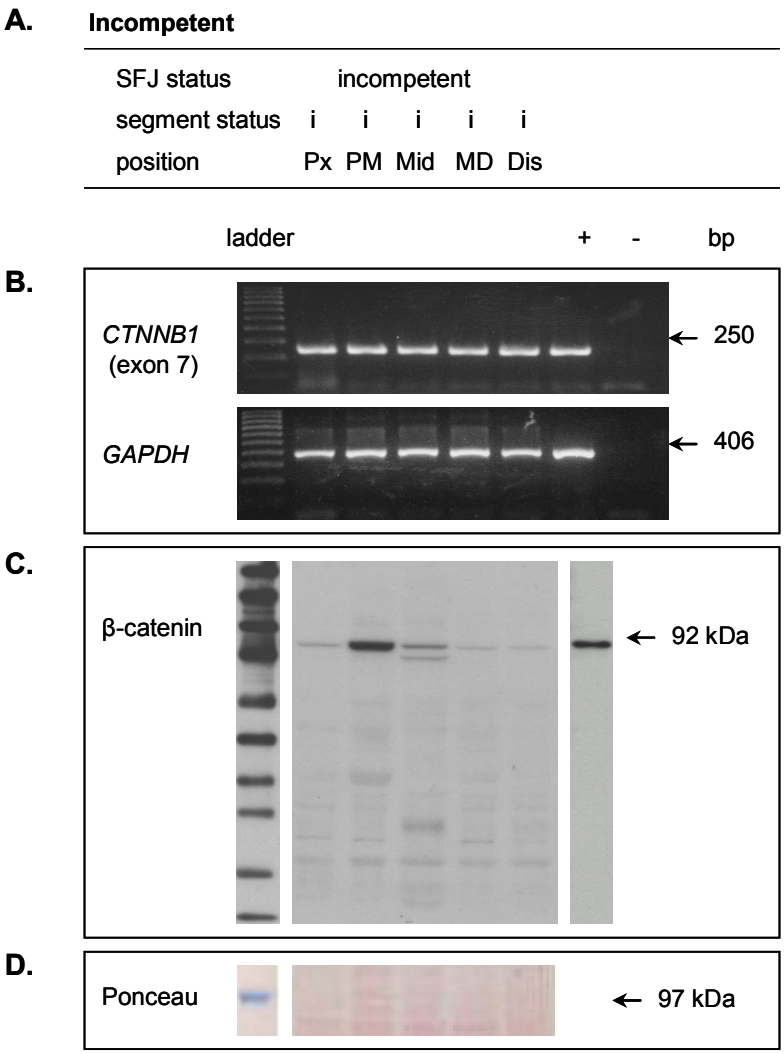


Figure 3.18 β-catenin gel and western blot of VVs sections with an incompetent SFJ. For each individual segment analysed, the competency of the SFJ and vessel wall was determined from duplex scan and classified as being competent with no reflux (c) or incompetent with reflux evident (i) (**A**). *β-catenin* gene (*CTNNB1*) transcription was examined by semi-quantitative RT-PCR. Products were visualised by agarose electrophoresis and the IOD of each band measured by scanning densitometry. Gene transcription was reported as IOD ratio of β-catenin PCR product normalised against control house keeping gene *GAP-3* (*GAPDH*) (**B**). β-catenin protein was detected with antibody and determined by western blot (**C**). β-catenin protein was measured by scanning densitometry and reported as IOD ratio of β-catenin protein normalised to total protein loaded (Ponceau S) (**D**).

3.3.4 **Results**

Patient demographics: GSV was obtained from 21 individuals resulting in a total of 99 and 87 samples available for gene and protein analysis respectively (Table 3.3). Of the 21 individuals examined, 16 had primary VVs as confirmed by venous duplex scan. Of these, 7 individuals presented with a functionally intact and competent SFJ (4 male, 3 female) resulting in 29 samples available for gene analysis and 30 samples for protein analysis respectively. The remaining 9 individuals with VVs presented with an incompetent SFJ (3 male, 6 female) resulting in 56 and 43 samples for gene and protein analysis respectively. Control GSV was obtained from 5 individuals (3 male, 2 female) making a total of 14 control samples available for both gene and protein analysis. RNA and protein of suitable quality was isolated from all vein sections analysed as illustrated in representative gels and western blot for individuals from control (Figure 3.16), competent SFJ (Figure 3.17) and those with an incompetent SFJ (Figure 3.18).

Varicose and control veins overall: For VVs overall, transcription of *β-catenin* was elevated compared to control vein (Figure 3.19). Similarly, the expression of β-catenin protein was elevated in VVs compared to control vein (Figure 3.19).

Competence at the sapheno-femoral junction: To examine the relevance of SFJ competency, the results were analysed specifically in conjunction with status of the SFJ (see Table 3.3). Transcription of *β-catenin* in VVs with competent SFJ was similar to that detected in control samples (Figure 3.20). However, when the SFJ was incompetent transcription of *β-catenin* was elevated compared to either control veins

Description	Varicose			Control
	competent SFJ	incompetent SFJ	total	
Individuals, *n* =	**7**	**9**	**16**	**5**
median age (range)	62 (28-69)	43 (27-58)	48 (27-56)*	63 (50-72)
male, *n* =	4	3	7	3
female, *n* =	3	6	9	2
mRNA samples, *n* =	**29**	**43**	**72**	**14**
male, *n* =	15	15	30	7
female, *n* =	14	28	42	7
Protein samples, *n* =	**30**	**39**	**69**	**14**
male, *n* =	15	15	30	7
female, *n* =	15	24	39	7

Table 3.3 Patient demographics and description of samples used in study.
Vein samples were obtained from 21 individuals. 16 patients had primary VVs (7 male, 9 female) resulting in a total of 72 and 69 samples available for gene and protein analysis respectively. Control GSV was obtained from 5 individuals (3 male, 2 female) resulting in a total of 14 control samples available for analysis. Overall, patients with VVs were younger than those from whom control veins were taken *p<0.030 patients with varicose versus control vein (Mann-Whitney U).

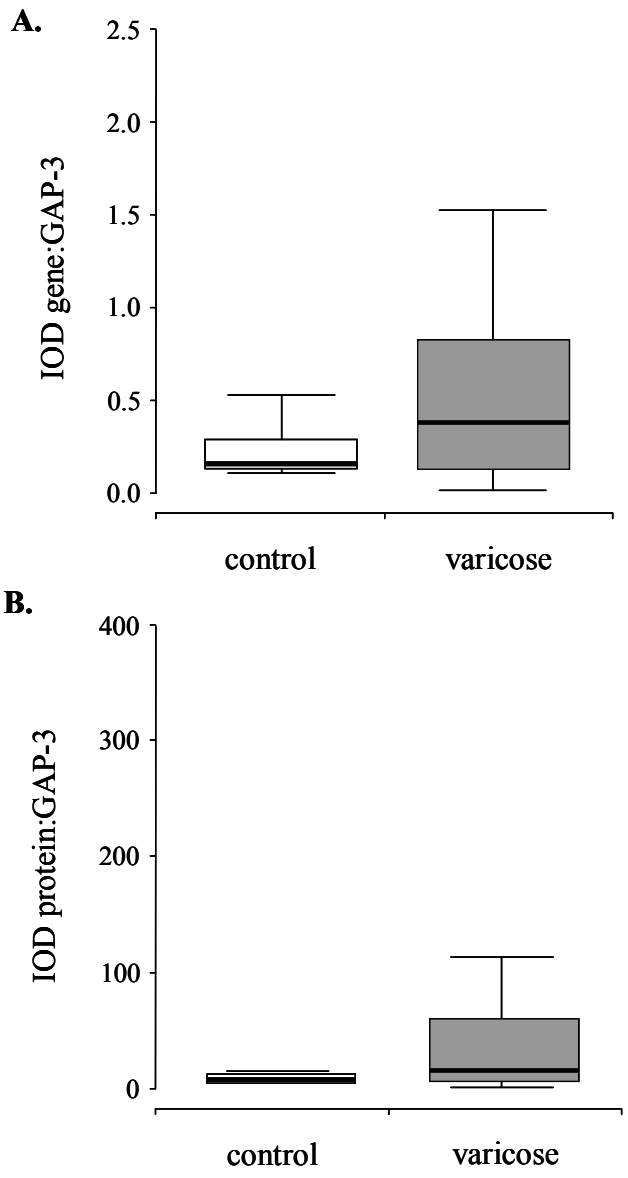


Figure 3.19 *β-catenin* transcription (**A**) and protein expression (**B**) in VVs overall. Control (open bars) and VVs overall (grey bars). Gene transcription is reported as a ratio of the IOD of *β-catenin* PCR product to corresponding product for *GAP-3*, determined by scanning densitometry. Expression of β-catenin protein reported as IOD ratio of β-catenin protein (western blot) to total protein (Ponceau S) determined by scanning densitometry. Box plots represent median values with interquartile range, error bars the range.

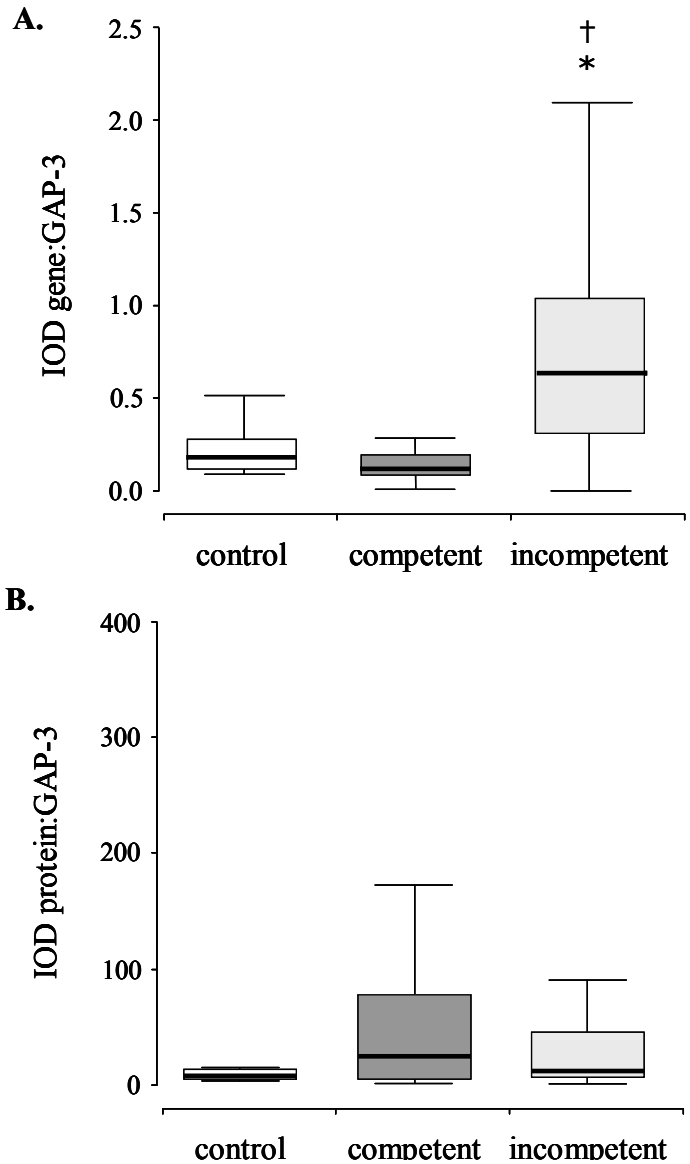


Figure 3.20 *β-catenin* transcription (**A**) and protein expression (**B**) by SFJ.
Sections of control veins (open bars), VVs from individuals with a competent (dark gray bars), or incompetent SFJ (light gray bars). Box plots represent median values with interquartile range, error bars the range, **p*<0.001 incompetent versus competent SFJ, **p*<0.002 incompetent versus control (Kruskal-Wallis).

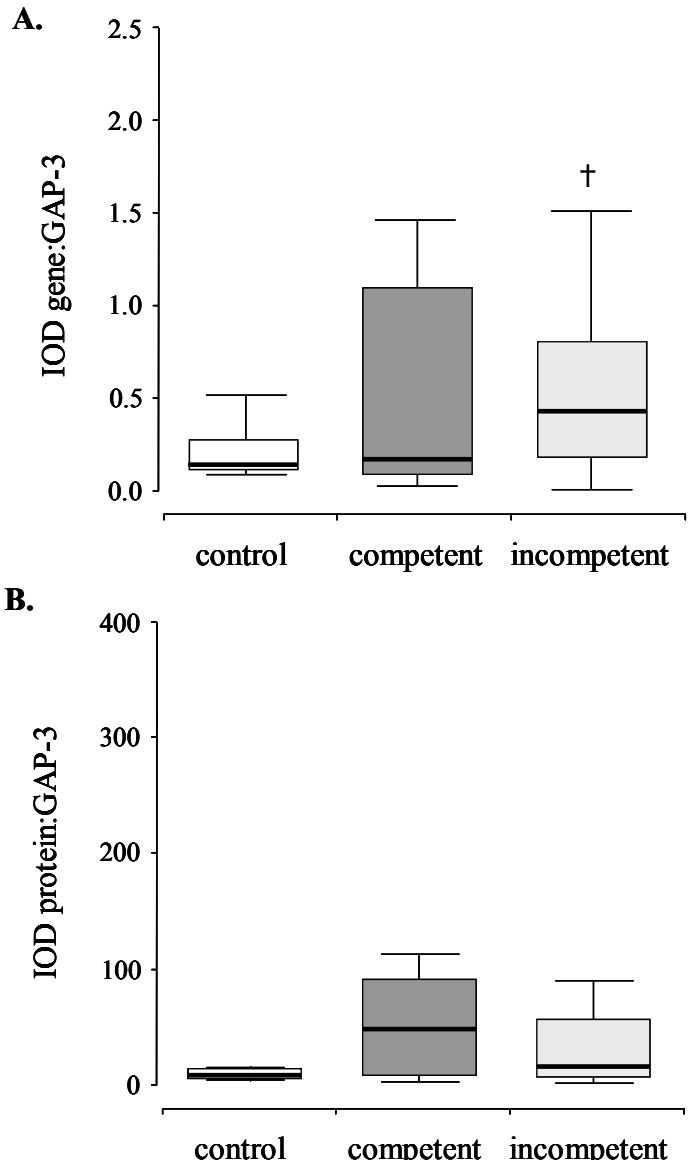


Figure 3.21 *β-catenin* transcription (**A**) and protein expression (**B**) by segment. Sections of control veins (open bars), and VVs where the section of vein wall was determined from duplex scan as competent (dark gray bars), or incompetent and showing reflux (light gray bars). Box plots represent median values with interquartile range, error bars the range, † $p<0.020$ versus control (Kruskal-Wallis).

(p<0.002) or VVs with a competent SFJ (p<0.001). The pattern of expression of β-catenin protein was different. Now, VVs with a competent SFJ, β-catenin protein was elevated compared to either control or VVs when the SFJ was incompetent (Figure 3.20). Interestingly, there was a negative (although not significant) correlation between *β-catenin* transcription and protein expression with SFJ incompetence.

Competence of the vein wall: To examine levels of gene transcription in conjunction with vein wall competence, results were now grouped and analysed as described in chapter 3.1.4. As with the SFJ, *β-catenin* transcription was elevated in sections of VVs that were incompetent compared to sections from either control (p<0.020) or competent sections of VVs (Figure 3.21). β-catenin protein expression was elevated in sections of VVs with competent vein wall segments compared to either control or segments that were incompetent (Figure 3.21). An inverse correlation, although not significant was observed between *β-catenin* transcription and protein expression in competent vein segments.

Position and competence of the sapheno-femoral junction: The transcription and expression of β-catenin was examined according to position from where derived; proximal, proximal-mid, mid, mid-distal and distal from the SFJ. For VVs overall, increased transcription of *β-catenin* correlated with descending position from the SFJ (p<0.050, Kendall's bivariate correlation; Figure 3.22). A similar (although not significant) correlation was seen for elevated expression of β-catenin protein (Figure 3.22). When considering both position and competency of the SFJ, transcription of *β-catenin* was unaffected by descending position in veins with a competent SFJ. Indeed levels of transcription were similar along the length of the vein (Figure 3.23).

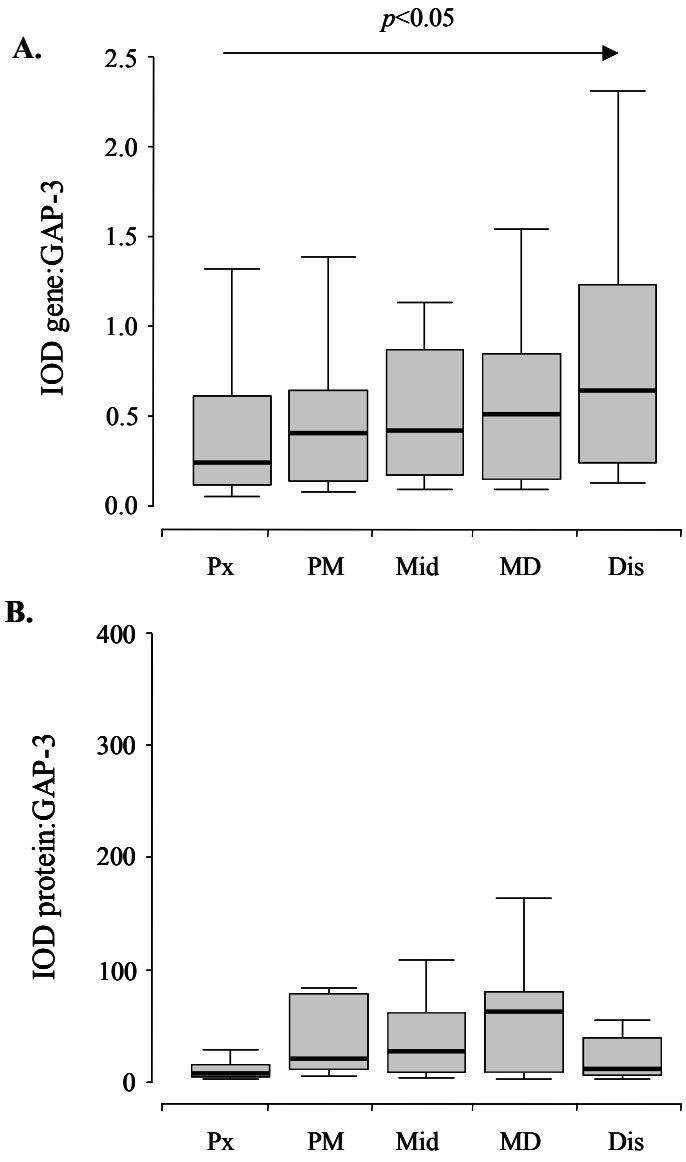


Figure 3.22 *β-catenin* transcription (**A**) and protein expression (**B**) by position.
Box plots represent median values with interquartile range, error bars the range, position descending from the SFJ as (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal, $p<0.050$, Kendall's bivariate correlation.

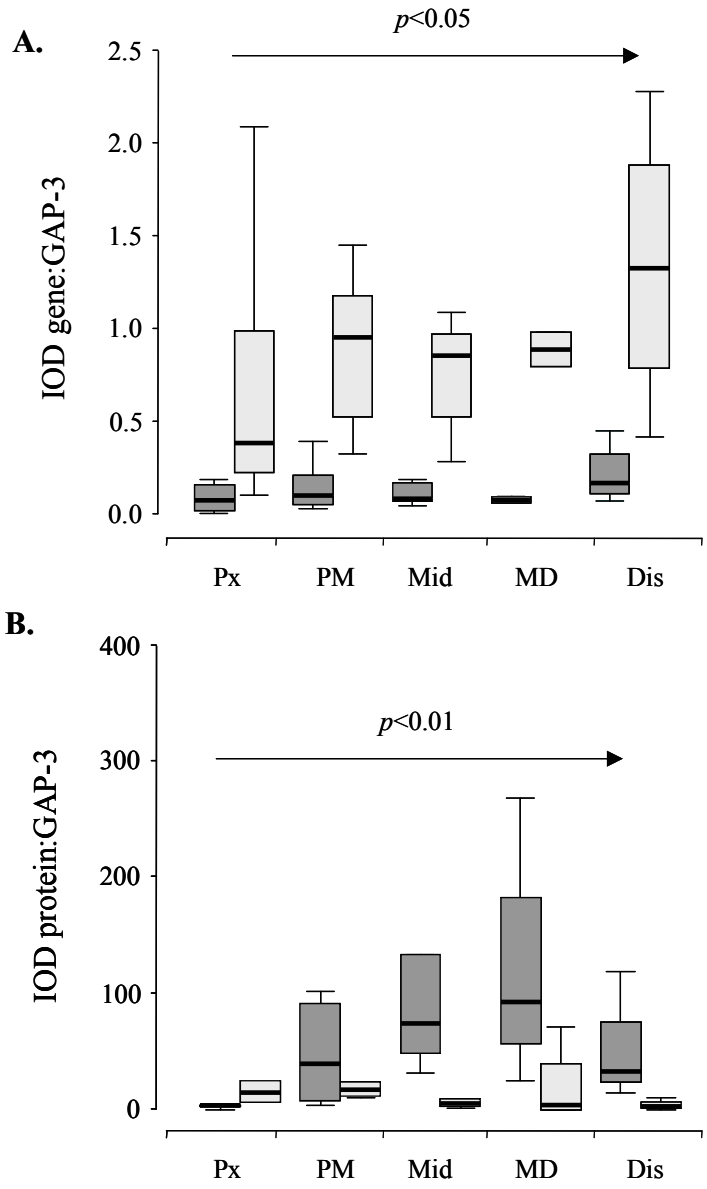


Figure 3.23 *β-catenin* transcription (**A**) and expression (**B**) by position & SFJ.
Box plots represent median values with interquartile range, error bars the range, position descending from a competent (dark grey bars) or incompetent (light grey bar) the SFJ as (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal. Gene transcription, incompetent SFJ $p<0.050$, protein expression, competent SFJ $p< 0.010$, (both Kendall's bivariate correlation).

In contrast when the SFJ was incompetent, *β-catenin* transcription was elevated for all segments along the vein length. Furthermore, there was a positive correlation for increased transcription of *β-catenin* with descending position from the SFJ ($p<0.050$, Kendall's bivariate correlation). The converse was detected for β-catenin protein, which was elevated when the SFJ was competent for all positions except proximal (Figure 3.23). In contrast β-catenin protein was similar for all anatomical positions in VVs samples with an incompetent SFJ. Notably, the elevated β-catenin protein correlated to descending position, away from the SFJ in VVs samples with a competent SFJ ($p< 0.010$, Kendall's bivariate correlation).

Transcription of *c-myc* and *cyclin D1*: To examine the relevance of elevated β-catenin here, the transcription of two nuclear target genes of β-catenin, *c-myc* and *cyclin D1* were similarly investigated (He *et al.,* 1998; Beherens *et al.,* 1996). In sections from VVs with an incompetent SFJ, transcription of both *c-myc* and *cyclin D1* were elevated compared to either control ($p<0.001$), or VVs with a competent SFJ ($p<0.001$, Figure 3.24). When considering segmental competency however, transcription of *c-myc* and *cyclin D1* were elevated in all VVs segments compared to control, *i.e.* when the segment was competent (*cyclin D1*, $p<0.050$) or incompetent (both $p<0.001$, Figure 3.25). Transcription of *β-catenin* correlated to that of *c-myc* and *cyclin D1* when the SFJ or segment was incompetent ($p>0.050$, Kendall's bivariate correlation). In contrast, an inverse relationship between β-catenin protein and *c-myc cyclin D1* transcription was observed with SFJ incompetence ($p>0.050$, Kendall's bivariate correlation). Elevated transcription of *c-myc* and *cyclin D1* correlated with decreasing position below an incompetent SFJ (*c-myc* $p<0.050$, *cyclin D1* $p<0.001$, Kendall's bivariate correlation, Figure 3.26).

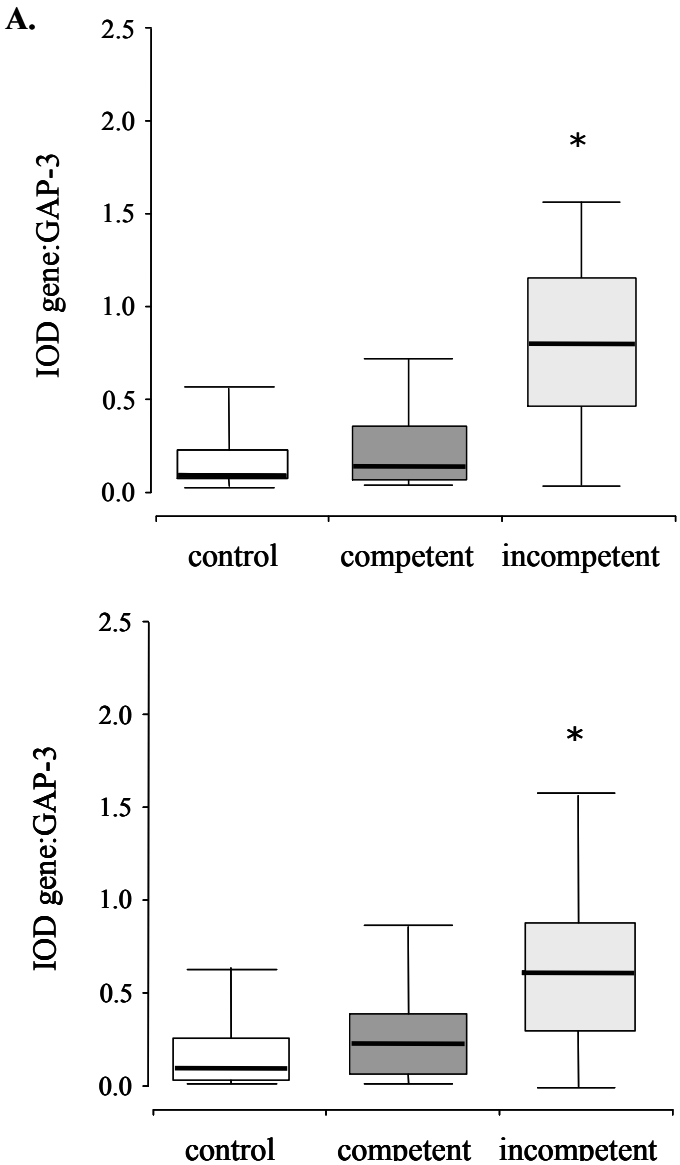


Figure 3.24 Transcription of *c-myc* (**A**) and *cyclin D1* (**B**) with SFJ status.
Sections of vein from control (open bars) and VVs from GSV with a competent (dark grey bars) or incompetent (light grey bars) SFJ. Box plots represent median values with interquartile range, error bars the range, *p<0.001 incompetent versus control and competent (Kruskal-Wallis).

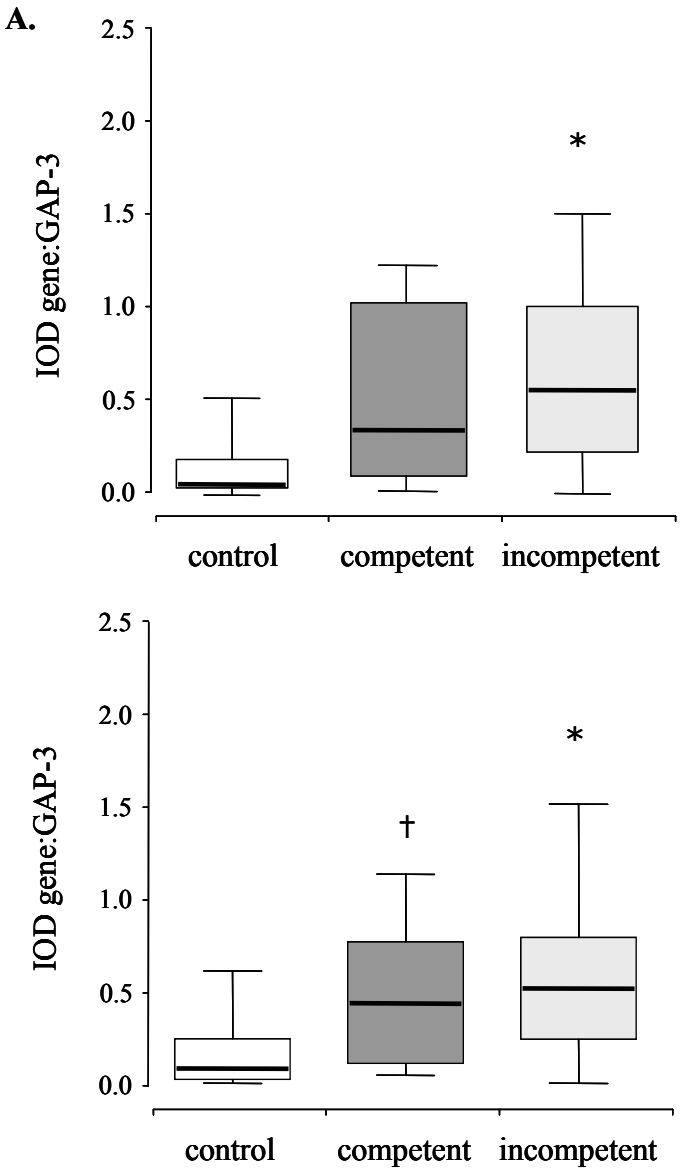


Figure 3.25 Transcription of *c-myc* (**A**) and *cyclin D1* (**B**) by segment status. Sections of vein from control (open bars) and VVs from GSV with a competent (dark grey bars) or incompetent (light grey bars) SFJ. Box plots represent median values with interquartile range, error bars the range, *p<0.001 incompetent versus control, $^{\dagger}p$<0.050 versus control (Kruskal-Wallis).

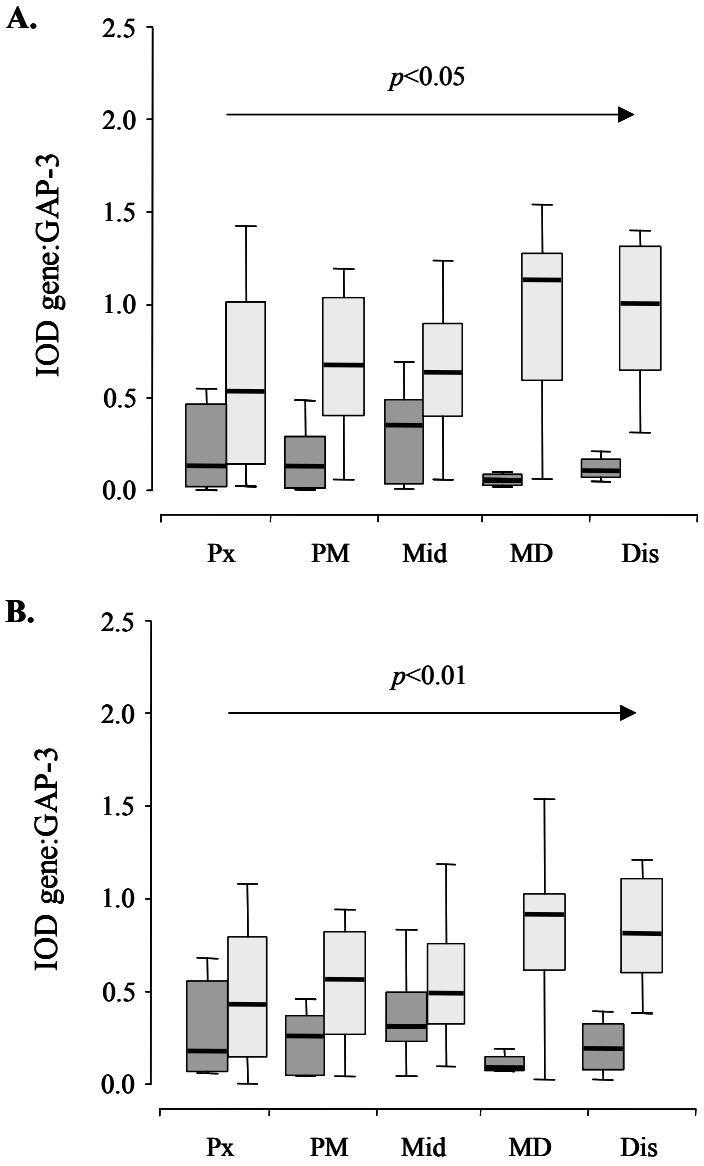


Figure 3.26 Transcription of *c-myc* (**A**) and *cyclin D1* (**B**) by position & SFJ.
Box plots represent median values with interquartile range, error bars the range, position descending from a competent (dark gray bars) or incompetent (light gray bar) the SFJ as (Px) proximal, (PM) proximal-mid, (Mid) mid, (MD) mid-distal and (Dis) distal. Gene transcription; *c-myc* incompetent SFJ $p<0.050$, *cyclin D1* incompetent $p<0.010$ (Kendall's bivariate correlation).

3.3.5 **Discussion**

Binding of VEGF to its receptor, VEGFR2 tyrosine phosphorylates VE cadherin and β-catenin (Esser *et al.,* 1998; Cohen *et al.,* 1999) disrupting the endothelial AJ (Kevil *et al.,* 1998). Dismantling the AJ results in loss of cadherin mediated cell-cell adhesion and increased vessel wall permeability (Kevil *et al.,* 1998) with the release of β-catenin into the cytoplasm (Moon *et al.,* 2004; Nelson *et al.,* 2004). Under normal conditions cytoplasmic β-catenin is rapidly degraded (Aberle *et al.,* 1997; Moon *et al.,* 2004), however, several signalling pathways modulate β-catenin turnover rates and regulate β-catenin availability (Moon *et al.,* 2004; Vincent *et al.,* 2004). If stabilised, cytoplasmic β-catenin translocates to the nucleus and initiates the transcription of genes implicated in cellular proliferation and differentiation (He *et al.,* 1998; Beherens *et al.,* 1996; see chapter 1.3.1). VVs demonstrate loss of release of VEGF-A (Hollingsworth *et al.,* 2001A) and aberrant VEGF-A gene transcription at a stage prior to vessel wall incompetence (see chapter 3.1.4). VEGF dysfunction in VVs therefore, may affect the release of β-catenin predisposing to elevated protein, and so the potential for inappropriate gene expression involved with vessel wall remodelling.

In VVs overall, both *β-catenin* gene transcription and protein expression were elevated (Figure 3.19) suggesting β-catenin protein was accumulated (or not degraded) in VVs. Transcription (as for *VEGF-A* and its receptors previously, see chapter 3.1.5) was elevated with SFJ incompetence (Figure 3.20A) and segmental incompetence (Figure 3.21A). In contrast, accumulated β-catenin protein was observed in VVs sections prior to SFJ incompetence (Figure 3.20B) (*i.e.* when the SFJ was functional and without reflux) suggesting perhaps that incompetence of the

SFJ may represent a later stage of disease development to when the SFJ is functional. Interestingly, alterations to β-catenin expression may not be due to transcriptional activation of the *β-catenin* gene. For SFJ competency, when comparing *β-catenin* transcription to quantity of protein detected, minimal *β-catenin* transcription was proportional to a small quantity of detectable protein in control vein (Figure 3.27A). In contrast, a similar quantity of *β-catenin* transcription (to control) produced proportionally a larger quantity of protein in VVs with a competent SFJ (Figure 3.27B). However, with SFJ incompetence the proportions between *β-catenin* transcription and protein expression returned to that observed previously for control segments (Figure 3.27C). This suggests that alterations to the accumulation of β-catenin protein in VVs, occurs before disturbances to the flow of blood in the vessel becomes apparent, substantiating earlier observations (for *VEGF-A*, see chapter 3.1.5) that SFJ incompetence may represent a later stage of disease progression. Similarly, when considering segmental competence, elevated β-catenin protein was observed before vessel wall incompetence (Figure 3.21B), suggesting perhaps that changes in the availability of β-catenin protein (potentially due to VEGF-A signalling) may occur as an earlier event *i.e.* before disturbances to blood flow. Furthermore, the accumulation of β-catenin protein detected here, without a concomitant up regulation of the *β-catenin* gene transcription, suggests perhaps that the increased availability of β-catenin protein may be regulated at the post transcriptional level.

According to the canonical *Wnt* pathway, cytoplasmic β-catenin protein is targeted for degradation by a complex of serine/threonine kinases consisting of GSK-3β and CK1 in a scaffold complex of axin and APC (Aberle *et al.,* 1997; Moon *et al.,* 2004, see chapter 1.3.1).

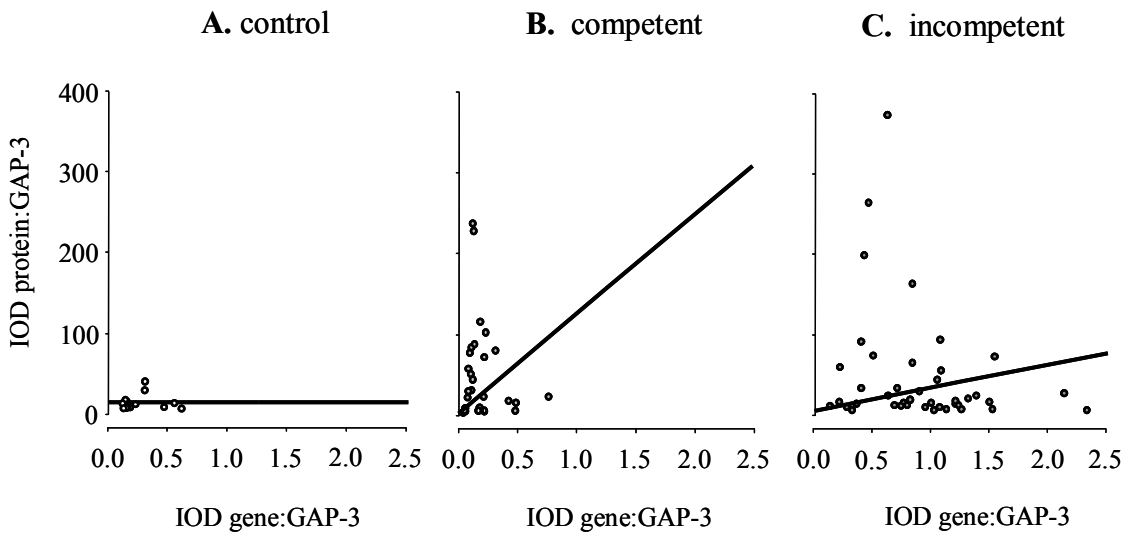


Figure 3.27 Proportional relationship between *β-catenin* transcription and protein. Influence of SFJ status on the relationship between *β-catenin* gene transcription (mRNA) and protein expression (protein). In control, low levels of mRNA produce minimal detectable β-catenin protein (**A**). However in VV, segments from veins with a competent SFJ, a higher proportion of protein per quantity of mRNA is produced (**B**). In VV with SFJ incompetence, elevated *β-catenin* transcription did not yield an associated elevated protein availability (**C**) suggesting perhaps that altered β-catenin activity may reflect an earlier event in varicogenesis, before disturbances to blood flow are observed.

Conventionally, β-catenin degradation is inhibited by Wnt signalling (Akiyama 2000) or mutation (Morin *et al.,* 1997), both of which prevent GSK-3β phosphorylation of β-catenin required for proteosome mediated degradation (Aberle *et al.,* 1997; Akiyama 2000; Moon *et al.,* 2004). In the canonical pathway, activation of the Frizzled family of receptors by Wnt ligands activates Dishevelled (Dsh) (Akiyama 2000; Goodwin *et al.,* 2002). Dsh inhibits GSK-3β which promotes β-catenin accumulation in the cytoplasm (Ikeda *et al.,* 1998; Sakanaka *et al.,* 1999; (see chapter 1.3.1). Alternatively, growth factor activation of PI3K-Akt or *Wnt*/Ca^{2+} pathway (Wnt activation of PKC) both inhibit GSK-3β resulting in elevated β-catenin (Chen *et al.,* 2000A; Kuhl *et al.,* 2000; Fukumoto *et al.,* 2001).

Although constitutively expressed in the vasculature (Cattelino *et al.*, 2003), β-catenin is rarely detected in normal adult vessels (Goodwin *et al.,* 2002; van Gijn *et al.,* 2002), but has been observed in the vascular endothelium in response to ischaemic injury (Blankesteijn *et al.,* 2001) and during vessel wall remodelling associated with angiogenesis (Eberhart *et al.,* 2001, Goodwin *et al.,* 2002; Wright *et al.,* 2002). However, in varicose GSV, vessel wall remodelling is predominately associated with proliferation and migration of SMC, not the endothelium (see chapter 1.1.6). Consequently, β-catenin has been implicated in the stabilisation of SMC cellular junctions (associated with N cadherin in SMC), and regulation of vascular SMC proliferation, migration and survival (Uglow *et al.,* 2003; Wang *et al.,* 2002; Slater *et al.,* 2004). Although the cellular origin of the β-catenin detected here is not known, the accumulation of β-catenin protein in VVs suggests an increased potential for nuclear translocation and β-catenin mediated gene transcription in the varicose vessel wall. Further studies investigating the cellular location of the β-catenin here

would be informative and may associate β-catenin expression with both cell type and remodelled area(s) of the VVs wall. Furthermore, as elevated β-catenin is normally associated with Wnt signalling and several studies demonstrate the presence of Wnt ligands and Fz receptors in vascular cells (Wright *et al.*, 1999; Goodwin *et al.*, 2002; van Gijn *et al.,* 2002) additional studies investigating the presence of Wnt ligands/receptors, and mutational status of β-catenin might help explain the elevated β-catenin protein observed here.

A further explanation for elevated β-catenin protein may be due to the dismantling of the AJ in response to growth factor activation like VEGF-A (Cohen *et al.*, 1999; Rahimi *et al.,* 1999; Lampugnani *et al.,* 1997; Dejana *et al.,* 2001). In addition to regulating endothelial barrier function (Cohen *et al.,* 1999), the AJ is suggested to regulate the activity of β-catenin by maintaining bound β-catenin in the AJ and thus preventing both its degradation (Aberle *et al.,* 1997) and translocation to the nucleus for gene transcription (Gottardi *et al.,* 2001; Dejana 2004). The accumulation of β-catenin protein in competent VV wall segments (*i.e* before disturbances to blood flow) may perhaps suggest the dismantling of the AJ in these segments (Wright *et al.*, 2002). In addition to releasing β-catenin however, dismantling of the AJ increases responsiveness of the vascular endothelium to growth factor signalling due to the loss of contact inhibition (Rahimi *et al.,* 1999; Lampugnani *et al.,* 1997A; see chapter 1.3.2). Notably, as a loss of contact inhibition is required for endothelial cell proliferation and migration associated with vessel remodelling (in response to both growth factor activation and disturbed blood flow) elevated β-catenin may indicate the initiation of the remodelling process (Rahimi *et al.,* 1999; Lampugnani *et al.,* 1997A; Dejana *et al* 2000; Dejana *et al.,* 2001).

With dismantling of the AJ following VEGF-A activation, VEGFR2 forms a complex with VE cadherin (Rahimi *et al.,* 1999; Wright *et al.,* 2002; Zanetti *et al.,* 2002) that localises to the intracellular junction (Carmeliet *et al.,* 1999; Rahimi *et al.,* 1999; Lampugnani *et al.,* 2003; see chapter 1.3.2). The formation of the VE cadherin VEGFR2 complex is dependant on the release of β-catenin (Carmeliet *et al.,* 1999) and furthermore modulates VEGFR2 signalling (Rahimi *et al.,* 1999; Zanetti *et al.,* 2002). Recruitment of adapter proteins (see chapter 1.2.5) to phosphorylated tyrosine residues on the intracellular VEGFR2 domain, determines the signalling pathway(s) activated (Carmeliet *et al.,* 1999; Lampugnani *et al.,* 2003). VEGF-A (via VEGFR2) induces activation of PI3K-Akt (Carmeliet *et al.,* 1999; Gerber *et al.,* 1998a) while simultaneously inhibiting PLCγ mediated proliferation in confluent EC (Lampugnani *et al.,* 2003). Conversely, when the AJ is dismantled, VEGF-A stimulates both PI3K-Akt and EC proliferation via PLCγ mediated MAPK activity (Gliki *et al*, 2002; Lampugnani *et al.,* 2003). Consequently, VEGF-A activation of PI3K-Akt via VEGFR2 not only inhibits EC apoptosis (Gerber *et al.,* 1998A; Shiojima *et al.,* 2002; see chapter 1.2.5) but potentially inhibits GSK-3β in a PI3K-Akt dependant manner (Chen *et al.,* 2000A; Fukumoto *et al.,* 2001). In terms of β-catenin therefore, VEGF-A binding to VEGFR2, in addition to releasing β-catenin from the AJ (Cohen *et al.*, 1999), may also regulate its stabilisation through PIK3-Akt mediated inhibition of GSK-3β (Chen *et al.,* 2000A; Fukumoto *et al.,* 2001; Gliki *et al*, 2002) resulting in the accumulated β-catenin observed here.

Besides regulating endothelial barrier function (Corada *et al,*. 1999), the endothelial AJ has been proposed as a mechanotransducer responsible for vessel wall responses to haemodynamic forces such as shear stress and disturbed blood flow (Noria *et al.,*

1999; Ukropec *et al.,* 2002; Shay-Salit *et al.,* 2002). In the arterial system it is generally accepted that the causative effect of haemodynamic forces like shear stress acting through the endothelium is chronic restructuring of blood vessels and the initiation of angiogenesis (Sho *et al.,* 2003). Although less is known of similar mechanisms in the venous system, in saphenous veins used as conduits in bypass grafting, the increased pressure and disturbed blood flow of the arterial system is associated with acute remodelling of the venous wall (Shay-Salit *et al.,* 2002; Sho *et al.,* 2003). Local disturbances in the flow of blood at sites along the length of the vein should stimulate the release of VEGF-A and VEGFR2 mediated disruption of the AJ, releasing of β-catenin (Shay-Salit *et al.,* 2002; Sho *et al.,* 2003). As venous incompetence tends to appear more distally in the leg, and at a position at, or below the underlying venous incompetence in the vein (Labropoulos *et al.,* 1999; Cooper *et al.,* 2003); investigations of the pattern of β-catenin expression in relation to position along the length of the vein, may show potential associations between disease presentation and β-catenin expression.

The position of the segment below the SFJ had a significant effect on the pattern of activity of β-catenin (Figure 3.22 and 3.23) with elevated β-catenin protein observed with descending position below a competent and functional SFJ (see Figure 3.23B). Again in VVs, elevated β-catenin protein was observed before disturbances to blood flow in vein with a competent and functional SFJ, suggesting perhaps that altered β-catenin activity may reflect an earlier event in varicogenesis. Notably, there was no change in the transcription of *VEGF-A* and its receptors with descending position, although the activation status of the VEGF receptors were not investigated. Additional studies should investigate the phosphorylation status of the VEGFR2

receptor as well as downstream mediators of VEGFR2 signalling (notably PI3K-Akt) which may help to clarify the potential role of VEGF-A signalling in the production/stabilisation of β-catenin seen here (Gliki *et al*, 2002).

Cytoplasmic β-catenin, if not degraded translocates to the nucleus (Huber *et al.,* 1996; Blankesteijn *et al.,* 2001), and initiates TCF/LEF transcription of numerous genes involved implicated in vessel wall remodelling including *c-myc*, *cyclin D1* and matrix metalloproteinases (Gumbiner 1995; Behrens *et al.,* 1996; He *et al.,* 1998; Shtutman *et al.,* 1999; see chapter 1.3.1). Consequently, to determine whether the elevated β-catenin detected here was functional and active, the transcription of the nuclear targets *c-myc* and *cyclin D1* were investigated (He *et al.,* 1998; Shtutman *et al.,* 1999). In VVs, transcription of both *c-myc* and *cyclin D1* were elevated with SFJ incompetence (Figure 3.24). However, when considering segmental competency, transcription of *c-myc* and *cyclin D1* were elevated in all VVs segments, *i.e.* when the segment was competent or incompetent (Figure 3.25), again, as for VEGFR2 (see chapter 3.1.4) and β-catenin protein previously, suggesting perhaps an earlier altered molecular event, before disturbances to blood flow become apparent.

Besides initiating the transcription of *c-myc* and *cyclin D1* in these samples, what role may β-catenin play in VVs before the onset of venous incompetence *i.e* may elevated β-catenin simply reflect a differing gene expression associated with an on-going pathological process. More recently, the model of a single molecular form of β-catenin that participates in cell adhesion and gene expression was suggested to be an over simplification of the mechanisms(s) involved (Gottardi *et al.,* 2004). Observations of multiple forms of β-catenin co-existing in the cell have introduced a

new layer of regulation, with differing conformations of β-catenin protein itself preferentially determining either adhesion or nuclear signalling roles (Gottardi *et al.,* 2004). As Wnt signalling was suggested to determine the β-catenin conformational change and binding specificity, further studies into the types of β-catenin molecular variations here maybe useful in explaining the elevated β-catenin observed. Alternatively, elevated β-catenin may simply be inducing expression of a different molecule (Akiyama 2000; Goodwin *et al.,* 2002). Indeed, besides inducing transcription of genes for proliferation, more recently β-catenin was demonstrated to elevate both the transcription and protein expression of VEGF-A *in vitro,* giving rise potentially to the possibility of another positive feedback loop involving VEGF-A (as for NO see chapter 1.2.6.2) in the vessel wall (Easwaran *et al.,* 2003; Skurk *et al.,* 2005).

As discussed previously, the results reported here should be interpreted with caution as they are based on small patient numbers. It would be more informative to extend these studies to more individuals, specifically those with an "earlier" stage of varicose disease (*i.e.* VVs with competent SFJ and/or vessel wall segments). Other problems with this sample set have been discussed (see chapter 3.1.5). A clearer understanding of the mechanisms underlying the development and progression of varicosity would help with the treatment of this disease, as disturbed β-catenin activity (possible due to VEGF) may precede vessel wall compromise in VVs, with elevated β-catenin protein potentially providing a mechanism for vessel wall remodelling.

Summary points

The results of this study can be summarised as follows:

1. The transcription and protein expression of β-catenin was elevated in sections of VVs compared to control.

2. The transcription of *β-catenin* was elevated in VVs when the SFJ was incompetent.

3. β-catenin protein was accumulated in competent segments of VVs *i.e.* before disturbances to the flow of blood.

4. β-catenin protein increased with descending position below a competent SFJ.

5. Altered activity of β-catenin may be associated in some part, with the process(es) of varicogenesis, representing an early event before development of vessel wall incompetence. In contrast SFJ incompetence may represent a later stage of disease development.

6. Elevated β-catenin protein availability may potentially drive vessel wall remodelling through the transcriptional activation of TCF/LEF targets, *c-myc* and *cyclin D1*.

SUMMARY AND CONCLUSIONS

4.1 SUMMARY AND CONCLUSIONS

In summary, how do the results presented here affect the hypothesis of a potential molecular abnormality in the VEGF-A system being involved in the initiation and development of primary VVs? If altered VEGF-A signalling is a driving force underlying the initiation and progression of venous incompetence and vessel remodelling in the vein, it may be expected that disturbances in the expression of VEGF-A and/or its receptors would associate with the pattern of disease presentation in the vein wall. Furthermore, the progressive nature of varicose disease (Jones *et al.,* 1999; Cooper *et al.,* 2003; Labropoulos *et al.,* 2005) together with the irregular distribution of the disease along the length of the vessel (Badier-Commader *et al.,* 2001; Wali *et al.,* 2001B), potentially may help to discriminate a differing gene activity between these sites; as determined by local disease severity and functional characteristics of varicose disease like disturbed blood flow (reflux/stasis) (Browse *et al.,* 1999; Nicolaides 2000).

Do varicose vessels have the capability to produce VEGF-A (and its receptors) and if so is altered transcription of *VEGF-A* and/or its receptors, a causative mechanism in VVs? The first study demonstrated that VVs have the capability to transcribe both *VEGF-A* and its receptors, and that gene activity of *VEGF-A* (and its receptors) was disturbed in VVs. Notably, transcription of *VEGF-A* and its membrane receptors were elevated before development of vessel wall incompetence, which may suggest an association in some part, with the process(es) of varicogenesis, potentially representing an altered early molecular event. In contrast however, incompetence of the SFJ may represent a later stage of disease development.

The second part of the hypothesis attempted to address whether the vessel wall could respond to, or control the VEGF-A produced appropriately. As VEGF-A mediates most of its dilatory and mitogenic effects through activation of VEGFR2 (Vaisman *et al.*, 1990; Millauer *et al.,* 1993; Waltenberger *et al.,* 1994), disordered functioning of VEGFR2 may play a role in the initiation and development of VVs. Two lines of investigation were examined. With VEGFR2 signal activation, the receptor may be redundant and not function correctly, or a continued signal may arise due to the inability to switch off, or deactivate the activated receptor.

In the vascular endothelium, the action of VEGFR2 is modulated by s.flt-1 (Kendall *et al.,* 1993; Barleon *et al.,* 1997B; Barleon *et al.,* 2001), which binds VEGF-A with high affinity and potentially modulates VEGF-A signalling (Siemester *et al.,* 1998B; Graubert *et al.,* 2001). The second study demonstrated that s.flt-1 was significantly elevated in plasma of patients with VVs. Notably, although baseline levels of s.flt-1 were elevated, there was a loss of response to venous hypertension in VVs, extending earlier observations suggesting a potential problem with VEGF-A responses in VVs.

If indeed signalling via VEGF-A (or receptors) is disturbed with the initiation of varicose disease, then alterations to VEGF-A's normal functions in regulating vascular permeability and dilation would similarly be affected. Although the mechanism(s) by which VEGF-A mediates permeability are not clearly understood, VEGF-A signalling dismantles the AJ resulting in a loss of endothelial cell adhesion, with increased paracellular permeability and the release of β-catenin to the cytoplasm (see chapters 1.2.6 and 1.3.2). If VEGF-A signalling is disturbed with the onset of varicose disease, which does indeed occur by a process of progressive "spreading

incompetence" (Jones *et al.,* 1999; Cooper *et al.,* 2003), then alterations to the pattern of expression of β-catenin may similarly associate to the patterns of disease presentation observed.

Study three demonstrated that β-catenin activity (as for VEGF-A and receptors previously) was disturbed in VVs. Furthermore, the pattern of activity of β-catenin expression was associated with the pattern of disease presentation, as determined by SFJ or vessel segment competency. This extends earlier observations that changes in the availability of β-catenin protein (potentially due to VEGF-A signalling) may represent an early event in the development of VVs, and be associated in some part, with the initiation of varicose disease. Furthermore, accumulated β-catenin protein potentially, may drive vessel wall remodelling through the transcriptional activation of TCF/LEF targets, *c-myc* and *cyclin D1*. However, as a large number of growth factors, including activation of the *Wnt* signalling pathway are known to stabilise β-catenin, the relevance of the association here between VEGF-A and β-catenin should be interpreted with caution, until additional studies can clarify their interactions.

The relationships described here are complex and due consideration needs to be given to a number of limitations with this study. Overall, all genes investigated were transcriptionally activated in VVs, and except for *s.flt-1* had a significant positive correlation to each other overall (see Table 4.1). Furthermore, the status of the SFJ had a significant effect on the transcriptional activation of all genes investigated, as illustrated by elevated transcription of all genes with SFJ incompetence. The strong association between venous incompetence and transcriptional activation was further illustrated by the significant partial correlations between genes when controlling for

both SFJ status (Table 4.2) and segmental competency (Table 4.3). The concern here therefore, may be that the transcriptional activation observed with venous incompetence may not be necessarily be associated with the pathological process of varicogenesis (blood reflux/stasis), but merely represent the results of a pan-transcriptional response by the components of the vessel wall to, for example an underlying chronic inflammation in the diseased vein. This seems unlikely however, as all gene transcripts were normalised against an internal cellular control *GAP-3*, which would also be elevated by a cellular pan-transcriptional response. In addition, the positive correlations observed are not associated with the transcription of all genes investigated (see Table 4.2 and 4.3, see for example *s.flt-1*). However, due to the mechanisms involved in the regulation of *s.flt-1* transcription (alternative splice variant, see Figure 2.4) it would be informative to investigate the transcription of other gene(s) in the vessel wall not necessarily associated with varicose disease or inflammation, for clarification.

In addition, the relationship between gene transcription and quantities of actual protein produced, need to be carefully investigated (as illustrated by the negative relationship between *β-catenin* gene transcription and protein expression, see Table 4.2). A clearer appreciation of the relationships between *VEGF-A/VEGFR* gene transcription and protein expression, together with a better understanding of the post transcriptional mechanisms controlling VEGF-A/VEGFR protein production in the vessel wall would be informative in determining the effects of disturbed VEGF-A function in the development of VVs. Finally, the analyses undertaken represent merely a "molecular snapshot" in time and may not fully appreciate the genetic and molecular nuances required to control VEGF-A activity. As such, additional studies

investigating the differing patterns of disease association to other molecules affected by and potentially affecting VEGF-A action in sections of vein at different stages of disease progression (varicose but competent to grossly varicose and incompetent) may further help our understanding of the potential role of VEGF-A in the initiation and development of varicose disease.

	V121	*V165*	*KDR*	*FLT*	*sflt1*	*βcat*	βcat WB	*myc*	*cyclin*
V121		+	+	+		+		+	+
V165	+		+	+		+		+	+
KDR	+	+		+		+		+	+
FLT	+	+	+			+		+	+
s.flt1									
βcat	+	+	+	+				+	+
βcat WB									
myc	+	+	+	+		+			+
cyclin	+	+	+	+		+		+	

Table 4.1 Bivariate correlations between all genes investigated in this study. Excluding *sflt*-1 and β-catenin protein there was a positive correlation between the transcription of all genes in the study (+ positive correlation, $p<0.010$, Kendall's bivariate correlation).

SFJ	*V121*	*V165*	*KDR*	*FLT*	*s.flt-1*	*βcat*	βcat WB	*myc*	*cyclin*
V121		++		++		++		++	+
V165	++		++	++		++		++	+
KDR		++		++	-	+		+	+
FLT	++	++	++			++		++	+
s.flt1			-						
βcat	++	++	+	++			-*	++	++
βcat WB						- *		-	
myc	++	++	+	++		++	-		++
cyclin	++	++	+	++		++		++	

Table 4.2 Partial correlations between all genes controlling for SFJ status.

A positive partial correlation between transcription of *VEGF-A,* the VEGF receptors, *β-catenin*, *c-myc* and *cyclin D1* (all $p<0.001$, Kendall's bivariate correlation) was demonstrated, when controlling for SFJ. Similiarly, there was a positive correlation between the transcription of *β-catenin* and that of *c-myc* and *cyclin D1* (all $p<0.001$, Kendall's bivariate correlation). In contrast, a negative correlation was found when examining *β-catenin* transcription and the amount of β-catenin protein detected ($p<0.010$, Kendall's bivariate correlation). (+ positive correlation, $p<0.010$; ++ positive correlation, $p<0.001$; - negative correlation, $p<0.050$; -* negative correlation, $p<0.010$, all Kendall's bivariate correlation).

SEG	*V121*	*V165*	*KDR*	*FLT*	*s.flt-1*	*βcat*	βcat WB	*myc*	*cyclin*
V121		++	++	++		++		++	++
V165	++		++	++		++		++	++
KDR	++	++		++	-	++		++	++
FLT	++	++	++			++		++	++
s.flt1			-						
βcat	++	++	++	++				++	++
βcat WB									
myc	++	++	++	++		++			++
cyclin	++	++	++	++		++		++	

Table 4.3 Partial correlations between all genes controlling for segment.

A positive partial correlation between transcription of *VEGF-A,* the VEGF receptors, *β-catenin*, *c-myc* and *cyclin D1* (all $p<0.001$, Kendall's bivariate correlation) was found, when controlling for segment. Similiarly increased transcription of *β-catenin* correlated to increased transcription of *c-myc* and *cyclin D1* (all $p<0.001$, Kendall's bivariate correlation). (++ positive correlation, $p<0.001$; - negative correlation, $p<0.050$, all Kendall's bivariate correlation).

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PUBLICATIONS

Publications

Hollingsworth, S., **Powell**, **G**., Barker S. (2005). PRIMARY VARICOSE VEINS: elevated plasma levels of the soluble isoform of the VEGF receptor flt-1 (s.flt-1). *Phlebology* **20(3)**, 117-122.

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Abstracts

Powell. **G**., Cooper, D. and Hollingsworth, S. (2005). PRIMARY VARICOSE VEINS: increased β-catenin availability may precede development of vessel wall compromise, and so provide a target for intervention. *Phlebology* **20(2),** 101.

Hollingsworth, S., **Powell**, **G**. and Barker, S (2003). VARICOSE VEINS: elevated plasma levels of the soluble isoform of the VEGF receptor Flt-1. *Phlebology* **18**, 48.

Presentations

Powell. **G**., Cooper, D. and Hollingsworth, S. (2005). PRIMARY VARICOSE VEINS: increased β-catenin availability may precede development of vessel wall compromise, and so provide a target for intervention. *Venous Forum Annual Spring Meeting, Brighton UK.*

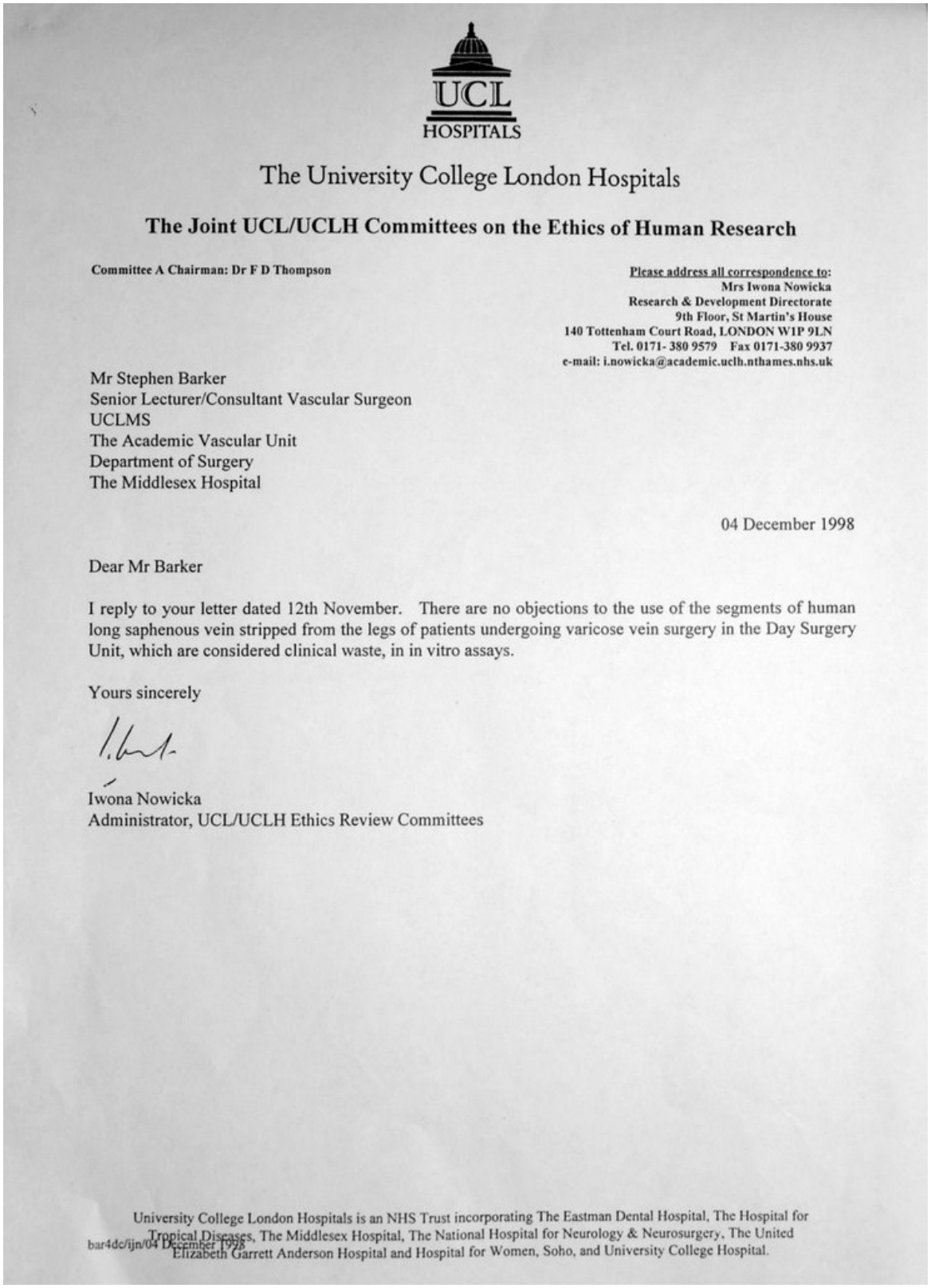
Hollingsworth, S., **Powell**, **G**., Barker, S. and Cooper, D (2003). PRIMARY VARICOSE VEINS: Altered transcription of VEGF and its receptors (KDR, Flt-1, soluble Flt-1) with sapheno-femoral junction incompetence. *European Society for Vascular Surgery (ESVS), Dublin UK.*

Hollingsworth, S., **Powell**, **G**., Barker, S (2003). VARICOSE VEINS: elevated plasma levels of the soluble isoform of the VEGF receptor Flt-1. *Venous Forum Annual Spring Meeting, Newcastle UK.*

APPENDICES

7.1 APPENDIX 1: ETHICS APPROVAL

7.1.1 Approval for use of stripped varicose veins



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The Joint UCL/UCLH Committees on the Ethics of Human Research

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Mr Stephen Barker
Senior Lecturer/Consultant Vascular Surgeon
UCLMS
The Academic Vascular Unit
Department of Surgery
The Middlesex Hospital

04 December 1998

Dear Mr Barker

I reply to your letter dated 12th November. There are no objections to the use of the segments of human long saphenous vein stripped from the legs of patients undergoing varicose vein surgery in the Day Surgery Unit, which are considered clinical waste, in in vitro assays.

Yours sincerely

Iwona Nowicka
Administrator, UCL/UCLH Ethics Review Committees

bar4dc/ijn/04 December 1998

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FAXED AND POSTED 21/2/01

20th February2001

Dear Mrs Nowicka,

In the past we have been granted permission to use both segments of human long saphenous vein, obtained at operation where they were waste products ie stripping of varicose veins, and human umbilical vein from umbilical cord following delivery, in *in vitro* assays; please see attached letters.

Our experimentation recently is involved with oestrogens and their receptors. It is likely that umbilical vein responds differently to other vein when exposed to oestrogens. To overcome the problem of poor 'quality' vein from varicose veins and 'atypical' vein from human umbilical artery, a ready supply of vein is unused off cuts from coronary artery bypass grafting, which are disposed of as clinical waste. Some groups, I gather, are already using this source within UCL.

We would be grateful therefore for clarification that permission to use vein that is a waste product would include that left over from bypass grafting. In addition, in light of the recent issues surrounding consent for use of tissues for research purposes should we be actively gaining written consent from the patients?

I look forward to your reply.

With kindest regards.

Yours sincerely,

David G Cooper MA(Hons) MBBChir MRCS
Clinical Research Fellow in Surgery

UCL
HOSPITALS

The University College London Hospitals

The Joint UCL/UCLH Committees on the Ethics of Human Research

Committee A Chairman: Dr F D Thompson

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Dr D G Cooper
Clinical Research Fellow in Surgery
Academic Vascular Unit
The Middlesex Hospital

Tuesday, March 20, 2001

Dear Dr Cooper

I reply to your letter dated 20th February on behalf of Dr Thomspon. He is happy for you to go ahead with the procedure detailed in your letter, however, suggests that you should obtain written consent from the patients.

Yours sincerely

Iwona Nowicka
Administrator, UCL/UCLH Ethics Review Committees

Telefax
To: D. Cooper
Fax: 020 7813 7971
From: Iwona Nowicka
Date: 27/03/01 Pages: 1

Cooper20mch/iin/Tuesday. March 20. 2001

TOTAL P.01

7.2 APPENDIX 2: RESULTS DATA TABLES

7.2.1 Sample characteristics

SampleID	sample	position	gender	age	leg	location	SFJ	segment
	Control							
1	**PN1a**		M	60	-	-	-	C
2	**1b**							C
3	**PN2a**		F	71	-	-	-	C
4	**2b**							C
5	**2c**							C
6	**PN3a**		M	50	-	-	-	C
7	**3b**							C
8	**PN4a**		M	72	-	-	-	C
9	**4b**							C
10	**4c**							C
11	**PN5a**		F	63	-	-	-	C
12	**5b**							C
13	**5c**							C
14	**5d**							C
	Competent SFJ							
15	**PC3**	Px	M	62	right	calf	C	C
16		PM						C
17		M						I
18		MD						I
19	**PC5**	Px	F	28	right	calf	C	I
20		PM						I
21		M						C
22		MD						C
23		Dis						C
24	**PC6**	Px	M	67	right	calf	C	I
25		PM						I
26		M						I
27	**PC7**	Px	F	69	right	thigh+calf	C	I
28		PM						I
29		M						I
30		MD						I
31		Dis						I
32	**PC8**	Px	M	62	right	thigh+calf	C	I
33		PM						I
34		M						I
35	**PC9**	Px	M	51	left	thigh+calf	C	I
36		PM						I
37		M						I
38		MD						I
39		Dis						I
40	**PC10**	Px	F	35		thigh+calf	C	I
41		PM						I
42		M						C
43		MD						C
44		Dis						C

SampleID	sample	position	gender	age	leg	location	SFJ	segment
	Incompetent SFJ							
45	**Pi 1**	Px	F	55	left	thigh+calf	I	I
46		PM						C
47		M						C
48		MD						C
49	**Pi 3**	Px	M	19	right	thigh+calf	I	I
50		PM						I
51		M						I
52		MD						I
53	**Pi 4**	Px	F	27	right	thigh+calf	I	I
54		PM						I
55		M						I
56		MD						I
57		Dis						I
58	**Pi 6**	Px	F	53	right	calf	I	I
59		PM						I
60		M						I
61		MD						I
62		Dis						I
63	**Pi 7**	Px	M	45	right	thigh+calf	I	I
64		PM						I
65		M						I
66		MD						I
67		Dis						I
68	**Pi 8**	Px	F	41	right	calf	I	I
69		PM						I
70		M						I
71		MD						I
72		Dis						C
73	**Pi 10**	Px	F	36	left	thigh+calf	I	I
74		PM						I
75		M						I
76		MD						I
77		Dis						C
78	**Pi 12**	Px	M	58	left	thigh+calf	I	I
79		PM						I
80		M						I
81		MD						I
82		Dis						I
83	**Pi 13**	Px	F	58	right	thigh+calf	I	I
84		PM						I
85		M						I
86		MD						I
87		Dis						I
88	**Pi 15**	Px	F	33	left	thigh+calf	I	I
89		PM						I
90		M						I
91		MD						I
92		Dis						C
93	**Pi 16**	Px	M	43	right	thigh+calf	I	I
94		PM						I
95		M						I
96		MD						I
97		Dis						C
98	**Pi 17**	Px	F	49	right	thigh+calf	I	I
99		PM						I
100		M						I
101		MD						I
102	**Pi 18**	Px	F	37	right	thigh+calf	I	C
103		PM						I
104		M						I
105		M-D						I
106		Dis						C
107	**Pi 19**	Px	-	-	-	-	I	
108		PM						
109		M						
110	**Pi 20**	Px	-	-	-	-	I	
111		PM						
112		M						

7.2.2 VEGF-A and VEGF receptor transcription

SampleID	sample	position	segment	VEGF 121	VEGF 165	KDR	flt-1	s.flt-1
	Control			**ratio**	**ratio**	**ratio**	**ratio**	**ratio**
1	**PN1a**		C	0.030	0.000	0.011	0.006	0.622
2	**1b**		C	0.023	0.000	0.010	0.007	0.656
3	**PN2a**		C	0.017	0.000	0.101	0.016	0.737
4	**2b**		C	0.029	0.000	0.153	0.117	0.753
5	**2c**		C	0.055	0.000	0.236	0.216	0.798
6	**PN3a**		C	0.000	0.000	0.000	0.043	0.590
7	**3b**		C	0.000	0.000	0.000	0.020	0.509
8	**PN4a**		C	0.014	0.000	0.000	0.000	0.611
9	**4b**		C	0.005	0.000	0.000	0.000	0.555
10	**4c**		C	0.033	0.000	0.000	0.000	0.561
11	**PN5a**		C	0.000	0.000	0.000	0.026	0.460
12	**5b**		C	0.000	0.000	0.000	0.012	0.564
13	**5c**		C	0.000	0.000	0.000	0.077	0.494
14	**5d**		C	0.000	0.000	0.000	0.007	0.528
***n* =**				14	14	14	14	14
	Competent SFJ			**ratio**	**ratio**	**ratio**	**ratio**	**ratio**
15	**PC3**	Px	C	0.066	0.000	0.098	0.034	0.640
16		PM	C	0.021	0.000	0.057	0.120	0.663
17		M	I	0.152	0.000	0.056	0.026	0.560
18		MD	I	0.073	0.000	0.088	0.064	0.444
19	**PC5**	Px	I	0.000	0.000	0.000	0.000	0.502
20		PM	I	0.000	0.000	0.000	0.000	0.644
21		M	C	0.000	0.000	0.000	0.000	0.683
22		MD	C	0.000	0.000	0.000	0.000	0.656
23		Dis	C	0.000	0.000	0.000	0.000	0.592
24	**PC6**	Px	I	0.000	0.000	0.000	0.000	0.596
25		PM	I	0.000	0.000	0.000	0.000	0.710
26		M	I	0.000	0.000	0.000	0.000	0.633
27	**PC7**	Px	I	0.060	0.029	0.188	0.016	0.229
28		PM	I	0.054	0.005	0.052	0.034	0.052
29		M	I	0.300	0.061	0.186	0.568	0.516
30		MD	I	**-**	**-**	**-**	**-**	**-**
31		Dis	I	0.248	0.039	0.369	0.225	0.782
32	**PC8**	Px	I	0.066	0.000	0.056	0.069	0.635
33		PM	I	0.036	0.000	0.002	0.010	0.640
34		M	I	0.144	0.000	0.005	0.015	0.663
35	**PC9**	Px	I	0.069	0.000	0.000	0.000	0.755
36		PM	I	0.057	0.000	0.026	0.003	0.696
37		M	I	0.013	0.000	0.000	0.000	0.883
38		MD	I	0.063	0.000	0.000	0.000	0.938
39		Dis	I	0.011	0.000	0.058	0.348	0.610
40	**PC10**	Px	I	0.041	0.000	0.045	0.047	0.516
41		PM	I	0.036	0.000	0.090	0.004	0.472
42		M	C	0.044	0.000	0.032	0.003	0.556
43		MD	C	0.070	0.000	0.060	0.001	0.588
44		Dis	C	0.087	0.000	0.081	0.005	0.578
***n* =**				29	29	29	29	29

SampleID	sample	position	segment	VEGF 121	VEGF 165	KDR	flt-1	s.flt-1
	Incompetent SFJ			**ratio**	**ratio**	**ratio**	**ratio**	**ratio**
45	**Pi 1**	Px	I	0.073	0.025	0.972	0.677	0.322
46		PM	C	0.081	0.040	1.016	0.433	0.249
47		M	C	0.142	0.069	1.179	0.729	0.472
48		MD	C	0.047	0.006	1.010	0.505	0.420
49	**Pi 4**	Px	I	0.057	0.000	0.359	0.311	0.699
50		PM	I	0.048	0.000	0.262	0.331	0.617
51		M	I	0.130	0.000	0.863	0.526	0.782
52		MD	I	0.142	0.000	0.533	0.413	0.753
53		Dis	I	0.029	0.000	0.331	0.360	0.711
54	**Pi 6**	Px	I	0.123	0.000	0.075	0.038	0.734
55		PM	I	0.130	0.000	0.297	0.173	0.797
56		M	I	0.285	0.047	0.132	0.118	0.659
57		MD	I	0.229	0.043	0.277	0.102	0.706
58		Dis	I	0.199	0.027	0.291	0.183	0.649
59	**Pi 7**	Px	I	0.219	0.078	0.061	0.486	0.563
60		PM	I	0.076	0.020	0.016	0.186	0.688
61		M	I	0.318	0.086	0.188	0.535	0.668
62		MD	I	0.204	0.037	0.031	0.264	0.835
63		Dis	I	0.303	0.068	0.081	0.403	0.670
64	**Pi 8**	Px	I	0.434	0.142	0.334	0.482	0.600
65		PM	I	0.302	0.068	0.085	0.100	0.669
66		M	I	0.177	0.028	0.271	0.147	0.593
67		MD	I	0.260	0.042	0.061	0.053	0.550
68		Dis	C	0.222	0.065	0.216	0.264	0.640
69	**Pi 10**	Px	I	0.246	0.086	0.711	0.393	0.583
70		PM	I	0.203	0.083	0.646	0.182	0.680
71		M	I	0.284	0.086	0.595	0.605	0.440
72		MD	I	0.301	0.065	0.497	0.533	0.482
73		Dis	C	0.311	0.138	0.554	0.427	0.757
74	**Pi 12**	Px	I	0.234	0.032	0.238	0.290	0.444
75		PM	I	0.285	0.029	0.176	0.256	0.548
76		M	I	0.229	0.064	0.276	0.112	0.565
77		MD	I	0.303	0.034	0.341	0.113	0.476
78		Dis	I	0.292	0.070	0.503	0.201	0.500
79	**Pi 13**	Px	I	0.291	0.106	0.802	0.627	0.601
80		PM	I	0.160	0.024	0.346	0.441	0.560
81		M	I	0.295	0.041	0.717	0.663	0.767
82		MD	I	0.182	0.020	0.552	0.286	0.663
83		Dis	I	0.256	0.041	0.753	0.319	0.608
84	**Pi 15**	Px	I	0.044	0.026	0.489	0.617	0.091
85		PM	I	0.110	0.105	0.387	0.419	0.466
86		M	I	0.195	0.079	0.329	0.553	0.607
87		MD	I	0.110	0.035	0.421	0.331	0.437
88		Dis	C	0.209	0.113	0.800	0.482	0.677
89	**Pi 16**	Px	I	0.035	0.022	0.115	0.299	0.066
90		PM	I	0.034	0.023	0.446	0.509	0.634
91		M	I	0.060	0.018	0.141	0.292	0.415
92		MD	I	0.203	0.019	0.231	0.936	0.670
93		Dis	C	0.026	0.013	0.111	0.201	0.299
102	**Pi 18**	Px	C	0.000	0.000	0.046	0.011	0.339
103		PM	I	0.000	0.000	0.207	0.012	0.668
104		M	I	0.000	0.000	0.082	0.101	0.645
105		M-D	I	0.000	0.000	0.006	0.008	0.750
106		Dis	C	0.000	0.000	0.026	0.136	0.850
n=				54	54	54	54	54

7.2.3 **s.flt-1 ELISA**

SampleID	Normal	Assay 1				Assay 2				Mean
	Arm	OD1	OD2	mean	**s.flt-1**	OD1	OD2	mean	**s.flt-1**	**s.flt-1**
1	**A002**	0.059	0.060	0.060	**37.45**	0.059	0.060	0.060	**37.45**	**37.45**
2	**A003**	0.083	0.075	0.079	**50.45**	0.082	0.070	0.076	**31.38**	**40.92**
3	**A005**	0.090	0.065	0.078	**49.45**	0.070	0.064	0.067	**24.46**	**36.95**
4	**A007**	0.069	0.063	0.066	**41.78**	0.076	0.070	0.073	**29.08**	**35.43**
5	**A009**	0.059	0.064	0.062	**38.78**	0.062	0.064	0.063	**21.38**	**30.08**
6	**A010**	0.077	0.066	0.072	**45.45**	0.063	0.066	0.065	**22.54**	**33.99**
7	**A011**	0.064	0.065	0.065	**40.78**	0.062	0.066	0.064	**22.15**	**31.47**
	***n* =**									7
	Leg before	OD1	OD2	mean	**s.flt-1**	OD1	OD2	mean	**s.flt-1**	**s.flt-1**
8	**A002**	0.033	0.068	0.051	**41.33**	0.067	0.080	0.074	**29.46**	**35.40**
9	**A003**	0.040	0.046	0.043	**33.00**	0.075	0.082	0.079	**33.31**	**33.15**
10	**A005**	0.044	0.056	0.050	**40.78**	0.080	0.077	0.079	**33.31**	**37.04**
11	**A007**	0.031	0.027	0.029	**17.44**	0.102	0.053	0.078	**32.54**	**24.99**
12	**A009**	0.046	0.047	0.047	**36.89**	0.074	0.070	0.072	**28.31**	**32.60**
13	**A010**	0.048	0.041	0.045	**34.67**	0.089	0.083	0.086	**39.08**	**36.87**
14	**A011**	0.042	0.052	0.047	**37.44**	0.065	0.068	0.067	**24.08**	**30.76**
	***n* =**									7
	Leg after	OD1	OD2	mean	**s.flt-1**	OD1	OD2	mean	**s.flt-1**	**s.flt-1**
15	**A002**	0.064	0.051	0.058	**49.11**	0.080	0.081	0.081	**34.85**	**41.98**
16	**A003**	0.082	0.099	0.091	**85.78**	0.160	0.142	0.151	**89.08**	**87.43**
17	**A005**	0.042	0.038	0.040	**29.67**	0.083	0.079	0.081	**35.23**	**32.45**
18	**A007**	0.050	0.063	0.057	**48.00**	0.091	0.090	0.091	**42.54**	**45.27**
19	**A009**	0.055	0.050	0.053	**43.56**	0.079	0.082	0.081	**34.85**	**39.20**
20	**A010**	0.064	0.057	0.061	**52.44**	0.098	0.104	0.101	**50.62**	**51.53**
21	**A011**	0.062	0.062	0.062	**54.11**	0.081	0.079	0.080	**34.46**	**44.29**
	***n* =**									7

SampleID	Varicose	Assay 1				Assay 2				Mean
	Arm	OD1	OD2	mean	**s.flt-1**	OD1	OD2	mean	**s.flt-1**	**s.flt-1**
22	**B007**	0.075	0.131	0.103	**46.47**	0.063	0.063	0.063	**19.80**	**33.13**
23	**B008**	0.071	0.070	0.071	**24.80**	0.068	0.070	0.069	**23.80**	**24.30**
24	**B009**	0.074	0.080	0.077	**29.13**	0.087	0.075	0.081	**31.80**	**30.47**
25	**B010**	0.080	0.078	0.079	**30.47**	0.198	0.068	0.133	**66.47**	**48.47**
26	**B011**	0.097	0.084	0.091	**38.13**	0.075	0.080	0.078	**29.47**	**33.80**
27	**B013**	0.086	0.091	0.089	**36.80**	0.083	0.082	0.083	**32.80**	**34.80**
28	**B014**	0.086	0.082	0.084	**33.80**	0.087	0.085	0.086	**35.13**	**34.47**
29	**B015**	0.121	0.109	0.115	**54.47**	0.086	0.101	0.094	**40.13**	**47.30**
30	**B016**	0.073	0.075	0.074	**27.13**	0.068	0.075	0.072	**25.47**	**26.30**
31	**B017**	0.077	0.084	0.081	**31.47**	0.077	0.087	0.082	**32.47**	**31.97**
32	**B018**	0.094	0.105	0.100	**44.13**	0.101	0.103	0.102	**45.80**	**44.97**
33	**B019**	0.118	0.126	0.122	**59.13**					**29.57**
34	**B020**	0.077	0.088	0.083	**32.80**	0.077	0.089	0.083	**33.13**	**32.97**
	***n* =**									13
	Leg before	OD1	OD2	mean	**s.flt-1**	OD1	OD2	mean	**s.flt-1**	**s.flt-1**
35	**B007**	0.048	0.080	0.064	**56.33**	0.065	0.065	0.065	**22.92**	**39.63**
36	**B008**	0.063	0.067	0.065	**57.44**	0.089	0.092	0.091	**42.54**	**49.99**
37	**B009**	0.074		0.074	**67.44**	0.094	0.090	0.092	**43.69**	**55.57**
38	**B010**	0.063	0.080	0.072	**64.67**	0.082	0.086	0.084	**37.54**	**51.10**
39	**B011**	0.060	0.074	0.067	**59.67**	0.081	0.089	0.085	**38.31**	**48.99**
40	**B013**	0.069	0.046	0.058	**49.11**	0.074	0.075	0.075	**30.23**	**39.67**
41	**B014**	0.079	0.091	0.085	**79.67**	0.130	0.133	0.132	**74.08**	**76.87**
42	**B015**	0.069	0.078	0.074	**66.89**	0.105	0.096	0.101	**50.23**	**58.56**
43	**B016**	0.060	0.073	0.067	**59.11**	0.087	0.087	0.087	**39.85**	**49.48**
44	**B017**	0.095	0.094	0.095	**90.22**	0.117	0.133	0.125	**69.08**	**79.65**
45	**B018**	0.084	0.083	0.084	**78.00**	0.113	0.112	0.113	**59.46**	**68.73**
46	**B019**	0.107	0.115	0.111	**108.56**	0.119	0.134	0.127	**70.23**	**89.39**
47	**B020**	0.066	0.070	0.068	**60.78**	0.086	0.090	0.088	**40.62**	**50.70**
	***n* =**									13
	Leg after	OD1	OD2	mean	**s.flt-1**	OD1	OD2	mean	**s.flt-1**	**s.flt-1**
48	**B007**	0.068	0.047	0.058	**49.11**	0.062	0.061	0.062	**20.23**	**34.67**
49	**B008**	0.057	0.071	0.064	**56.33**	0.083	0.090	0.087	**39.46**	**47.90**
50	**B009**	0.062	0.067	0.065	**56.89**	0.108	0.118	0.113	**59.85**	**58.37**
51	**B010**	0.077	0.088	0.083	**76.89**	0.084	0.089	0.087	**39.46**	**58.18**
52	**B011**	0.066	0.062	0.064	**56.33**	0.075	0.082	0.079	**33.31**	**44.82**
53	**B013**	0.073	0.067	0.070	**63.00**	0.088	0.088	0.088	**40.62**	**51.81**
54	**B014**	0.086	0.082	0.084	**78.56**	0.157	0.149	0.153	**90.62**	**84.59**
55	**B015**	0.082	0.108	0.095	**90.78**	0.131	0.126	0.129	**71.77**	**81.27**
56	**B016**	0.080	0.074	0.077	**70.78**	0.099	0.101	0.100	**49.85**	**60.31**
57	**B017**	0.073	0.087	0.080	**74.11**	0.104	0.110	0.107	**55.23**	**64.67**
58	**B018**	0.084	0.093	0.089	**83.56**	0.114	0.125	0.120	**64.85**	**74.20**
59	**B019**	0.139	0.178	0.159	**161.33**	0.156	0.152	0.154	**91.38**	**126.36**
60	**B020**	0.076	0.076	0.076	**69.67**	0.096	0.101	0.099	**48.69**	**59.18**
	***n* =**									13

7.2.4 β-catenin transcription

SampleID	sample	position	segment	β cat 7	β cat 7	β catenin 7	β cat 11	mean
	Control			**ratio 1**	**ratio 2**	**mean**	**ratio 1**	**ratio**
1	**PN1a**		C	0.302	0.192	0.247	0.035	**0.141**
2	**1b**		C	0.087	0.136	0.111	**-**	**0.111**
3	**PN2a**		C	0.378	0.111	0.245	0.041	**0.143**
4	**2b**		C	0.253	0.233	0.243	0.111	**0.177**
5	**2c**		C	0.157	0.128	0.142	**-**	**0.142**
6	**PN3a**		C	0.326	0.175	0.251	0.025	**0.138**
7	**3b**		C	0.163	0.143	0.153	**-**	**0.153**
8	**PN4a**		C	0.621	0.414	0.518	0.069	**0.293**
9	**4b**		C	0.323	0.276	0.300	**-**	**0.300**
10	**4c**		C	0.935	0.650	0.793	0.129	**0.461**
11	**PN5a**		C	0.450	0.364	0.407	0.036	**0.222**
12	**5b**		C	0.632	0.443	0.538	**-**	**0.538**
13	**5c**		C	0.583	0.627	0.605	**-**	**0.605**
14	**5d**		C	0.117	0.120	0.118	**-**	**0.118**
n =						14		14
	Competent SFJ			**ratio 1**	**ratio 2**	**mean**	**ratio 1**	**ratio**
15	**PC3**	Px	C	0.055	0.035	0.045	**-**	**0.045**
16		PM	C	0.163	0.032	0.097	**-**	**0.097**
17		M	I	0.116	0.034	0.075	**-**	**0.075**
18		MD	I	0.353	0.060	0.207	**-**	**0.207**
19	**PC5**	Px	I	0.813	0.135	0.474	**-**	**0.474**
20		PM	I	0.151	0.076	0.113	0.501	**0.307**
21		M	C	0.276	0.081	0.178	**-**	**0.178**
22		MD	C	0.137	0.074	0.105	**-**	**0.105**
23		Dis	C	0.209	0.060	0.134	0.220	**0.177**
24	**PC6**	Px	I	0.215	**-**	0.215	**-**	**0.215**
25		PM	I	0.114	**-**	0.114	0.728	**0.421**
26		M	I	0.323	**-**	0.323	1.197	**0.760**
27	**PC7**	Px	I	0.034	0.074	0.054	**-**	**0.054**
28		PM	I	0.086	0.193	0.140	0.006	**0.073**
29		M	I	0.351	0.074	0.213	**-**	**0.213**
30		MD	I	**-**	**-**	-	**-**	**-**
31		Dis	I	0.713	0.945	0.829	0.133	**0.481**
32	**PC8**	Px	I	0.071	0.251	0.161	**-**	**0.161**
33		PM	I	0.065	0.265	0.165	**-**	**0.165**
34		M	I	0.073	0.157	0.115	**-**	**0.115**
35	**PC9**	Px	I	0.024	0.040	0.032	**-**	**0.032**
36		PM	I	0.035	0.073	0.054	**-**	**0.054**
37		M	I	0.064	0.087	0.075	**-**	**0.075**
38		MD	I	0.097	0.087	0.092	**-**	**0.092**
39		Dis	I	0.151	0.054	0.102	**-**	**0.102**
40	**PC10**	Px	I	0.212	0.221	0.216	**-**	**0.216**
41		PM	I	0.084	0.174	0.129	**-**	**0.129**
42		M	C	0.100	0.128	0.114	**-**	**0.114**
43		MD	C	0.085	0.160	0.123	**-**	**0.123**
44		Dis	C	0.079	0.119	0.099	0.349	**0.224**
n =						29		29

SampleID	sample	position	segment	β cat 7	β cat 7	β catenin 7	β cat 11	mean
	Incompetent SFJ			**ratio 1**	**ratio 2**	**mean**	**ratio 1**	**ratio**
45	**Pi 1**	Px	I	0.791	0.677	0.734	**-**	**0.734**
46		PM	C	1.044	0.783	0.914	2.060	**1.487**
47		M	C	0.943	0.567	0.755	1.481	**1.118**
48		MD	C	**-**	**-**	-	**-**	**-**
49	**Pi 3**	Px	I	0.191	0.064	0.128	**-**	**0.128**
50		PM	I	0.446	0.267	0.356	**-**	**0.356**
51		M	I	0.429	0.198	0.314	**-**	**0.314**
52		MD	I	**-**	**-**	-	**-**	
53	**Pi 4**	Px	I	0.032	0.582	0.307	**-**	**0.307**
54		PM	I	0.040	0.137	0.089	0.875	**0.482**
55		M	I	0.267	0.595	0.431	**-**	**0.431**
56		MD	I	0.168	0.744	0.456	**-**	**0.456**
57		Dis	I	0.227	0.355	0.291	0.609	**0.450**
58	**Pi 6**	Px	I	0.126	0.286	0.206	**-**	**0.206**
59		PM	I	1.197	0.700	0.949	1.032	**0.990**
60		M	I	1.252	0.868	1.060	**-**	**1.060**
61		MD	I	1.243	0.789	1.016	**-**	**1.016**
62		Dis	I	1.306	0.550	0.928	3.709	**2.318**
63	**Pi 7**	Px	I	0.795	0.040	0.417	**-**	**0.417**
64		PM	I	0.795	1.245	1.020	0.236	**0.628**
65		M	I	0.735	1.148	0.942	**-**	**0.942**
66		MD	I	0.566	1.092	0.829	**-**	**0.829**
67		Dis	I	0.686	0.974	0.830	2.202	**1.516**
68	**Pi 8**	Px	I	1.283	2.974	2.128	**-**	**2.128**
69		PM	I	1.211	3.061	2.136	0.616	**1.376**
70		M	I	0.866	0.482	0.674	**-**	**0.674**
71		MD	I	1.176	0.403	0.789	**-**	**0.789**
72		Dis	C	1.048	0.086	0.567	1.831	**1.199**
73	**Pi 10**	Px	I	1.577	1.045	1.311	**-**	**1.311**
74		PM	I	1.054	1.030	1.042	**-**	**1.042**
75		M	I	1.097	1.041	1.069	0.709	**0.889**
76		MD	I	1.206	1.241	1.224	**-**	**1.224**
77		Dis	C	1.096	1.207	1.152	1.345	**1.248**
78	**Pi 12**	Px	I	1.244	1.156	1.200	**-**	**1.200**
79		PM	I	1.088	0.498	0.793	0.443	**0.618**
80		M	I	1.154	0.986	1.070	**-**	**1.070**
81		MD	I	0.881	0.785	0.833	**-**	**0.833**
82		Dis	I	1.161	0.898	1.029	0.486	**0.757**
93	**Pi 16**	Px	I	0.835	0.573	0.704	**-**	**0.704**
94		PM	I	0.760	0.560	0.660	0.256	**0.458**
95		M	I	0.842	0.786	0.814	**-**	**0.814**
96		MD	I	2.154	0.919	1.536	**-**	**1.536**
97		Dis	C	0.916	0.713	0.814	1.336	**1.075**
98	**Pi 17**	Px	I	0.175	0.134	0.154	**-**	**0.154**
99		PM	I	0.100	0.069	0.085	**-**	**0.085**
100		M	I	0.072	0.066	0.069	**-**	**0.069**
101		MD	I	0.105	0.031	0.068	**-**	**0.068**
102	**Pi 18**	Px	C	0.394	0.143	0.269	**-**	**0.269**
103		PM	I	0.494	0.347	0.420	0.361	**0.391**
104		M	I	0.332	0.449	0.391	**-**	**0.391**
105		M-D	I	0.447	0.539	0.493	**-**	**0.493**
106		Dis	C	0.175	0.397	0.286	0.140	**0.213**
107	**Pi 19**	Px		0.027	0.022	0.024	**-**	**0.024**
108		PM		0.398	0.355	0.377	**-**	**0.377**
109		M		0.557	0.051	0.304	**-**	**0.304**
110	**Pi 20**	Px		0.056	0.050	0.053	**-**	**0.053**
111		PM		0.054	0.051	0.052	**-**	**0.052**
112		M		0.327	0.481	0.404	**-**	**0.404**
n =						56		56

7.2.5 β-catenin protein expression

SampleID	sample	position	segment	β-catenin	ponceau S	β-catenin	ponceau S	mean
	Control			**IOD 1**	**IOD 1**	**IOD 2**	**IOD 2**	**ratio**
1	**PN1a**		C	18.2	1.54	**-**	**-**	**11.84**
2	**1b**		C	51.7	6.09	**-**	**-**	**8.49**
3	**PN2a**		C	12.1	3.37	**-**	**-**	**3.60**
4	**2b**		C	24.9	5.73	**-**	**-**	**4.34**
5	**2c**		C	12.9	2.46	**-**	**-**	**5.23**
6	**PN3a**		C	44.7	3.28	**-**	**-**	**13.63**
7	**3b**		C	22.3	3.81	**-**	**-**	**5.86**
8	**PN4a**		C	115.0	4.46	**-**	**-**	**25.78**
9	**4b**		C	95.4	2.65	**-**	**-**	**36.01**
10	**4c**		C	16.0	3.72	**-**	**-**	**4.30**
11	**PN5a**		C	40.3	4.59	**-**	**-**	**8.79**
12	**5b**		C	10.9	1.23	**-**	**-**	**8.84**
13	**5c**		C	5.8	2.54	**-**	**-**	**2.28**
14	**5d**		C	9.0	2.06	**-**	**-**	**4.38**
n =								14
	Competent SFJ			**IOD 1**	**IOD 1**	**IOD 2**	**IOD 2**	**ratio**
15	**PC3**	Px	C	15.9	4.67	**-**	**-**	**3.41**
16		PM	C	215.4	4.57	**-**	**-**	**47.13**
17		M	I	80.5	3.05	**-**	**-**	**26.39**
18		MD	I	49.7	2.40	**-**	**-**	**20.69**
19	**PC5**	Px	I	8.6	3.53	**-**	**-**	**2.44**
20		PM	I	322.9	4.22	**-**	**-**	**76.52**
21		M	C	346.5	3.10	**-**	**-**	**111.77**
22		MD	C	91.3	1.13	**-**	**-**	**80.79**
23		Dis	C	18.3	2.84	**-**	**-**	**6.46**
24	**PC6**	Px	I	4.5	3.23	**-**	**-**	**1.39**
25		PM	I	67.4	4.62	**-**	**-**	**14.58**
26		M	I	48.1	2.35	**-**	**-**	**20.46**
27	**PC7**	Px	I	4.7	3.10	**-**	**-**	**1.53**
28		PM	I	98.7	5.16	**-**	**-**	**19.13**
29		M	I	280.4	4.07	**-**	**-**	**68.90**
30		MD	I	193.2	1.14	**-**	**-**	**169.45**
31		Dis	I	59.8	5.03	**-**	**-**	**11.89**
32	**PC8**	Px	I	7.5	2.94	**-**	**-**	**2.57**
33		PM	I	12.6	4.84	**-**	**-**	**2.59**
34		M	I	101.9	2.48	**-**	**-**	**41.07**
35	**PC9**	Px	I	0.0	3.61	**-**	**-**	**0.00**
36		PM	I	20.5	3.46	**-**	**-**	**5.92**
37		M	I	155.7	2.86	**-**	**-**	**54.45**
38		MD	I	140.0	1.88	**-**	**-**	**74.48**
39		Dis	I	115.6	4.21	**-**	**-**	**27.46**
40	**PC10**	Px	I	16.1	4.81	**-**	**-**	**3.34**
41		PM	I	368.9	4.36	**-**	**-**	**84.60**
42		M	C	846.9	3.62	**-**	**-**	**233.94**
43		MD	C	461.6	2.05	**-**	**-**	**225.18**
44		Dis	C	251.2	2.53	**-**	**-**	**99.29**
n =					30			30

SampleID	sample	position	segment	β-catenin	ponceau S	β-catenin	ponceau S	mean
	Incompetent SFJ			**IOD 1**	**IOD 1**	**IOD 2**	**IOD 2**	**ratio**
45	**Pi 1**	Px	I	11.4	2.19	**-**	**-**	**5.19**
46		PM	C	6.0	2.55	19.0	1.05	**10.22**
47		M	C	0.0	2.90	10.0	3.13	**1.60**
48		MD	C	14.5	2.31	**-**	**-**	**6.27**
49	**Pi 3**	Px	I	8.3	1.67	**-**	**-**	**4.97**
50		PM	I	0.0	3.07	16.0	0.94	**8.51**
51		M	I	0.0	5.96	6.0	3.39	**0.88**
52		MD	I	0.6	2.86	**-**	**-**	**0.23**
58	**Pi 6**	Px	I	24.3	2.31	**-**	**-**	**10.53**
59		PM	I	2.0	4.24	27.0	1.43	**9.68**
60		M	I	9.0	3.09	9.0	2.06	**3.64**
61		MD	I	0.0	2.96	**-**	**-**	**0.00**
62		Dis	I	0.1	2.60	**-**	**-**	**0.04**
63	**Pi 7**	Px	I	459.7	2.36	**-**	**-**	**194.80**
64		PM	I	0.0	6.09	36.0	0.93	**19.35**
65		M	I	12.0	4.08	22.0	3.29	**4.81**
66		MD	I	229.2	3.86	**-**	**-**	**59.37**
67		Dis	I	3.6	2.34	**-**	**-**	**1.54**
68	**Pi 8**	Px	I	19.1	0.91	**-**	**-**	**20.95**
69		PM	I	6.0	5.08	57.0	1.56	**18.86**
70		M	I	20.0	5.08	29.0	2.81	**7.13**
71		MD	I	26.1	4.13	**-**	**-**	**6.33**
72		Dis	C	19.9	2.28	**-**	**-**	**8.74**
73	**Pi 10**	Px	I	15.0	1.06	**-**	**-**	**14.16**
74		PM	I	2.0	3.93	59.0	0.76	**39.07**
75		M	I	29.0	7.10	105.0	2.34	**24.48**
76		MD	I	25.1	3.73	**-**	**-**	**6.73**
77		Dis	C	7.1	5.86	**-**	**-**	**1.21**
78	**Pi 12**	Px	I	69.1	5.72	**-**	**-**	**12.07**
79		PM	I	446.0	6.67	618.0	0.92	**369.30**
80		M	I	354.0	13.29	429.0	2.84	**88.85**
81		MD	I	938.7	5.93	**-**	**-**	**158.29**
82		Dis	I	56.0	5.67	**-**	**-**	**9.88**
93	**Pi 16**	Px	I	143.4	5.24			**27.36**
94		PM	I	719.0	7.71	775.0	1.82	**259.54**
95		M	I	15.0	5.19	46.0	1.86	**13.81**
96		MD	I	303.5	4.49			**67.60**
97		Dis	C	206.4	4.13			**49.97**
102	**Pi 18**	Px	C	15.7	3.69			**4.26**
103		PM	I	88.0	4.86	171.0	1.12	**85.39**
104		M	I	28.0	4.48	115.0	2.35	**27.59**
105		M-D	I	284.7	4.16			**68.44**
106		Dis	C	232.3	4.24			**54.80**
n =					43			43

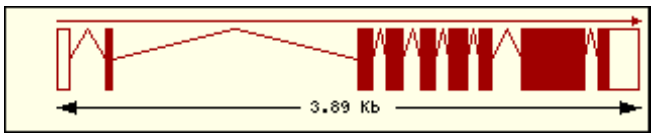
7.2.6 c-myc and cyclin D1 transcription

SampleID	sample	position	segment	c-myc 1	cmyc-2	mean	cyclin D1	cyclin D1	mean
	Control			**ratio 1**	**ratio 2**	**mean**	**ratio 1**	**ratio 2**	**mean**
1	**PN1a**		C	0.061	0.029	**0.045**	0.026	0.110	**0.068**
2	**1b**		C	0.034	0.073	**0.053**	0.001	0.111	**0.056**
3	**PN2a**		C	0.049	0.052	**0.050**	0.018	0.068	**0.043**
4	**2b**		C	0.085	0.022	**0.053**	0.021	0.023	**0.022**
5	**2c**		C	0.103	0.215	**0.159**	1.083	0.163	**0.623**
6	**PN3a**		C	0.044	0.082	**0.063**	0.117	0.149	**0.133**
7	**3b**		C	0.000	0.000	**0.000**	0.033	0.000	**0.016**
8	**PN4a**		C	0.062	0.016	**0.039**	0.081	0.006	**0.044**
9	**4b**		C	0.061	0.004	**0.032**	0.047	0.016	**0.031**
10	**4c**		C	0.099	0.077	**0.088**	0.095	0.264	**0.179**
11	**PN5a**		C	0.006	0.381	**0.194**	0.144	0.353	**0.248**
12	**5b**		C	0.552	0.509	**0.530**	0.539	0.370	**0.455**
13	**5c**		C	0.088	0.304	**0.196**	0.172	0.346	**0.259**
14	**5d**		C	0.070	0.369	**0.220**	0.061	0.182	**0.122**
n =						14			14
	Competent SFJ			**ratio 1**	**ratio 2**	**mean**	**ratio 1**	**ratio 2**	**mean**
15	**PC3**	Px	C	0.049	0.009	**0.029**	0.106	0.018	**0.062**
16		PM	C	0.023	0.021	**0.022**	0.116	0.022	**0.069**
17		M	I	0.014	0.018	**0.016**	0.176	0.282	**0.229**
18		MD	I	0.026	0.032	**0.029**	0.119	0.016	**0.067**
19	**PC5**	Px	I	0.113	0.481	**0.297**	0.309	0.441	**0.375**
20		PM	I	0.064	0.314	**0.189**	0.278	0.414	**0.346**
21		M	C	0.283	0.426	**0.355**	0.427	0.469	**0.448**
22		MD	C	0.029	0.129	**0.079**	0.104	0.088	**0.096**
23		Dis	C	0.026	0.185	**0.105**	0.130	0.118	**0.124**
24	**PC6**	Px	I	0.403	0.697	**0.550**	0.395	0.715	**0.555**
25		PM	I	0.281	0.695	**0.488**	0.272	0.645	**0.459**
26		M	I	0.605	0.782	**0.693**	0.730	0.945	**0.838**
27	**PC7**	Px	I	0.449	0.480	**0.464**	0.668	0.703	**0.685**
28		PM	I	0.125	0.463	**0.294**	0.576	0.158	**0.367**
29		M	I	0.008	0.113	**0.061**	0.370	0.245	**0.307**
30		MD	I	**-**	**-**	**-**	**-**	**-**	**-**
31		Dis	I	0.173	0.262	**0.217**	0.671	0.108	**0.389**
32	**PC8**	Px	I	0.013	0.003	**0.008**	0.030	0.303	**0.166**
33		PM	I	0.012	0.011	**0.011**	0.003	0.072	**0.037**
34		M	I	0.014	0.964	**0.489**	0.021	0.431	**0.226**
35	**PC9**	Px	I	0.067	0.005	**0.036**	0.100	0.000	**0.050**
36		PM	I	0.047	0.014	**0.031**	0.074	0.001	**0.038**
37		M	I	0.054	0.026	**0.040**	0.071	0.000	**0.035**
38		MD	I	0.025	0.060	**0.043**	0.076	0.052	**0.064**
39		Dis	I	0.029	0.076	**0.053**	0.026	0.005	**0.016**
40	**PC10**	Px	I	0.067	0.213	**0.140**	0.093	0.255	**0.174**
41		PM	I	0.065	0.209	**0.137**	0.084	0.426	**0.255**
42		M	C	0.405	0.511	**0.458**	0.463	0.525	**0.494**
43		MD	C	0.083	0.130	**0.107**	0.121	0.258	**0.190**
44		Dis	C	0.070	0.185	**0.127**	0.231	0.275	**0.253**
n =						29			29

SampleID	sample	position	segment	c-myc 1	cmyc-2	mean	cyclin D1	cyclin D1	mean
	Incompetent SFJ			**ratio 1**	**ratio 2**	**mean**	**ratio 1**	**ratio 2**	**mean**
45	**Pi 1**	Px	I	0.708	1.197	**0.953**	0.658	0.521	**0.589**
46		PM	C	0.849	1.525	**1.187**	0.913	0.955	**0.934**
47		M	C	0.704	1.285	**0.994**	0.789	0.689	**0.739**
48		MD	C	0.919	1.182	**1.051**	0.680	0.879	**0.779**
49	**Pi 3**	Px	I	0.034	0.954	**0.494**	0.036	0.282	**0.159**
50		PM	I	0.106	1.224	**0.665**	0.500	0.444	**0.472**
51		M	I	0.190	0.946	**0.568**	0.304	0.334	**0.319**
52		MD	I	1.211	1.121	**1.166**	0.935	0.894	**0.914**
53	**Pi 4**	Px	I	0.376	0.629	**0.503**	0.421	0.430	**0.426**
54		PM	I	0.292	0.852	**0.572**	0.303	0.318	**0.310**
55		M	I	0.869	0.670	**0.769**	0.817	0.779	**0.798**
56		MD	I	0.729	1.065	**0.897**	0.758	1.214	**0.986**
57		Dis	I	1.007	0.730	**0.869**	0.929	0.594	**0.761**
58	**Pi 6**	Px	I	0.163	0.875	**0.519**	0.258	0.174	**0.216**
59		PM	I	0.783	1.243	**1.013**	0.717	0.626	**0.672**
60		M	I	0.328	0.964	**0.646**	0.605	0.440	**0.523**
61		MD	I	1.225	1.854	**1.540**	1.190	1.341	**1.266**
62		Dis	I	1.347	1.364	**1.355**	1.305	1.091	**1.198**
63	**Pi 7**	Px	I	1.115	0.990	**1.053**	0.925	0.807	**0.866**
64		PM	I	0.635	1.449	**1.042**	0.699	0.866	**0.783**
65		M	I	0.814	1.348	**1.081**	0.576	0.948	**0.762**
66		MD	I	1.042	1.218	**1.130**	0.656	0.908	**0.782**
67		Dis	I	1.210	1.579	**1.395**	0.964	1.129	**1.046**
68	**Pi 8**	Px	I	1.329	1.515	**1.422**	1.354	0.789	**1.072**
69		PM	I	0.893	1.384	**1.139**	1.095	0.708	**0.902**
70		M	I	0.353	1.202	**0.777**	0.653	0.287	**0.470**
71		MD	I	0.699	1.807	**1.253**	1.094	0.722	**0.908**
72		Dis	C	1.084	1.175	**1.129**	1.400	0.892	**1.146**
73	**Pi 10**	Px	I	1.435	1.110	**1.273**	1.024	0.508	**0.766**
74		PM	I	1.075	0.556	**0.816**	0.850	0.842	**0.846**
75		M	I	1.065	1.396	**1.231**	1.115	1.242	**1.179**
76		MD	I	1.932	1.081	**1.507**	2.435	0.614	**1.525**
77		Dis	C	1.119	1.394	**1.257**	1.258	0.446	**0.852**
78	**Pi 12**	Px	I	0.760	0.823	**0.792**	1.061	0.553	**0.807**
79		PM	I	0.723	0.829	**0.776**	1.112	0.444	**0.778**
80		M	I	0.425	0.746	**0.585**	0.617	0.355	**0.486**
81		MD	I	0.475	0.678	**0.577**	0.740	0.475	**0.608**
82		Dis	I	0.959	0.739	**0.849**	0.940	0.512	**0.726**
93	**Pi 16**	Px	I	0.596	0.498	**0.547**	0.660	0.493	**0.576**
94		PM	I	0.241	0.655	**0.448**	0.121	0.378	**0.249**
95		M	I	0.328	0.586	**0.457**	0.344	0.307	**0.326**
96		MD	I	0.968	1.567	**1.268**	0.855	1.172	**1.013**
97		Dis	C	0.339	0.496	**0.418**	0.514	0.420	**0.467**
98	**Pi 17**	Px	I	0.008	0.000	**0.004**	0.023	0.002	**0.012**
99		PM	I	0.074	0.000	**0.037**	0.012	0.064	**0.038**
100		M	I	0.072	0.001	**0.036**	0.159	0.233	**0.196**
101		MD	I	0.077	0.003	**0.040**	0.012	0.033	**0.023**
102	**Pi 18**	Px	C	0.194	0.100	**0.147**	0.262	0.253	**0.258**
103		PM	I	0.607	0.438	**0.523**	0.659	0.465	**0.562**
104		M	I	0.790	0.451	**0.621**	0.696	0.375	**0.536**
105		M-D	I	0.331	0.183	**0.257**	0.244	0.283	**0.264**
106		Dis	C	0.371	0.219	**0.295**	0.437	0.322	**0.379**
107	**Pi 19**	Px		0.000	0.000	**0.000**	0.000	0.000	**0.000**
108		PM		0.495	0.143	**0.319**	0.372	0.185	**0.278**
109		M		0.511	0.100	**0.305**	0.453	0.485	**0.469**
110	**Pi 20**	Px		0.121	0.072	**0.097**	0.201	0.068	**0.135**
111		PM		0.058	0.047	**0.053**	0.137	0.006	**0.071**
112		M		0.099	0.044	**0.072**	0.147	0.039	**0.093**
n =						58			58

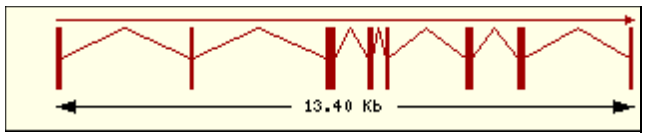
7.3 APPENDIX 3: GENE SEQUENCES

7.3.1 Glyceraldehyde 3-Phosphate Dehydrogenase (EC 1.2.1.12)



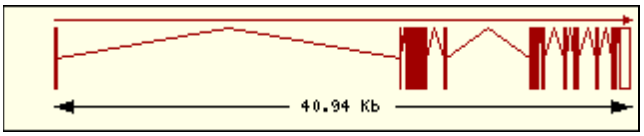
Gene	chromosome	**12p13**
	exons	9
	transcript length (bp)	1315
Protein	amino acids	335
	MW (kDa)	36
	Charge	8.00
	pI	8.73
Sequence	PCR product (bp)	406
		452

7.3.2 Vascular Endothelial Growth Factor (VEGF-A)



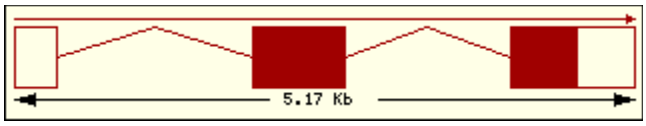
Gene	chromosome		**6p12**
	exons		8
	transcript length (bp)		784
Protein	amino acids		232
	MW (kDa)		27
	charge		21.50
	pI		9.08
Sequence	PCR product (bp)	vegf165	375
		vegf121	243

7.3.3 β-catenin (CTNNB1)



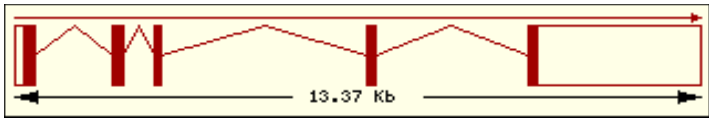
Gene	chromosome	**3p22**
	exons	16
	transcript length (bp)	3357
Protein	amino acids	781
	MW (kDa)	85
	charge	-11.5
	pI	5.71
Sequence	PCR product (bp)	Bcat7 250 βcat11 247

7.3.4 c- myc proto-oncogene protein (MYC)



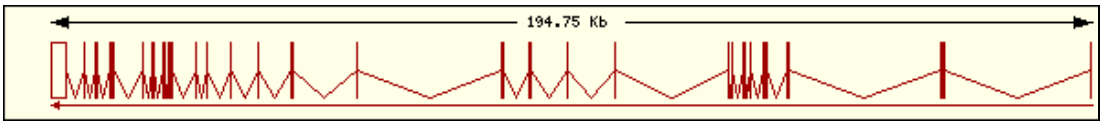
Gene	chromosome	**8**
	exons	3
	transcript length (bp)	2 168
Protein	amino acids	439
	MW (kDa)	48
	charge	-8.50
	pI	5.16
Sequence	PCR product (bp)	248

7.3.5 Cyclin D1 (CNND1)



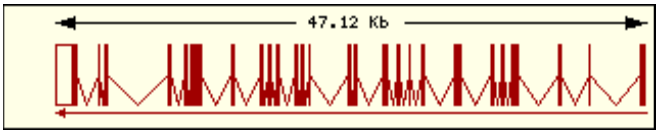
Gene	chromosome	**11**
	exons	5
	transcript length (bp)	4 288
Protein	amino acids	295
	MW (kDa)	33
	charge	-11.00
	pI	4.68
Sequence	PCR product (bp)	354

7.3.6 Vascular Endothelial Growth Factor Receptor 1 (flt-1) (EC 2.7.1.112)



Gene	chromosome	**13q12**
	exons	30
	transcript length (bp)	7 090
Protein	amino acids	1 338
	MW (kDa)	150
	charge	33.00
	pI	8.48
Sequence	PCR product (bp)	flt-1 248
		s.flt-1 119

7.3.7 Vascular Endothelial Growth Factor Receptor 2 (KDR) (EC 2.7.1.112)



Gene	chromosome	**4q12**
	exons	30
	transcript length (bp)	5 832
Protein	amino acids	1 356
	MW (kDa)	151
	charge	-14.00
	pI	5.64
Sequence	PCR product (bp)	312

7.4 APPENDIX 4: BUFFERS

Soltran Kidney perfusate (pH 7.1)	**per litre**
Potassium citrate	8.6 g
Sodium citrate	8.2 g
Mannitol	33.8 g
Magnesium Sulphate	10.0g

RT-PCR: 5x First Strand Buffer

250mM Tris-HCl, pH 8.3

375mM KCl

15mM $MgCl_2$

NuPAGE® MOPS-SDS Running Buffer pH 7.7 (20x)	**500ml**
50mM MOPS	104.6 g
50mM Tris base	60.6 g
0.1% SDS	10.0 g
1mM EDTA	3.0 g

NuPAGE® Transfer Buffer pH7.2 (20x)	**125ml**
25mM Bicine	10.2 g
25mM Bis-Tris (free base)	13.1 g
1mM EDTA	0.75 g

NuPAGE® LDS Sample Buffer pH 8.5 (4x)	**500ml**
106 mM Tris HCl	0.666 g
141 mM Tris base	0.682 g
2% LDS	0.800 g
10% Glycerol	4.000 g
0.51 mM EDTA	0.006 g
0.22 mM SERVA® Blue G250 (1% solution)	0.750 ml
0.175 mM Phenol Red (1% solution)	0.250 ml